BIONEXGEN Participants

No	Full Name	Short Name
1	University of Manchester (UK)	UNIMAN
2	Universitaet Stuttgart (Germany)	USTUTT
3	Danmarks Tekniske Universitaet (Denmark)	DTU
4	Mikrobiologicky Ustav – AVCR, V.V.I. (Czech Republic)	IMIC
5	Rijksuniversiteit Groningen (NL)	RUG
6	Clea Technologies BV (NL)	CLEA
7	EntreChem SL (Spain)	ENTR
8	Universidad de Oviedo (Spain)	UNIOVI
9	Galab Laboratories GMBH (Germany)	GALAB
10	Leibniz-Institut fur Pflanzenbiochemie (Germany)	IPB
11	ACIB GmbH (Austria)	ACIB
12	Kungliga Tekniska Hoegskolan (Sweden)	KTH
13	LentiKat's a.s. (Czech Republic)	LK
14	Slovenska Technicka Univerzita V Bratislave (Slovakia)	SUT
15	BASF SE (Germany)	BASF
16	University College London (UK)	UCL
17	Chemistry Innovation Ltd (UK)	CIKTN

PUBLISHABLE SUMMARY Please provide an executive summary. The length of this part cannot exceed 1 page

BIONEXGEN (Developing the Next Generation of Biocatalysts for Industrial Chemical Synthesis) was a Flagship EU collaborative research project that ran between the 1st February 2011 and 31st January 2014. The project brought together a consortium of 17 leading European companies and universities, with the overall goal to develop key enzyme classes and technologies for use in eco-efficient manufacturing processes in the chemical industries. Biocatalysis is the general term for the use of natural catalysts, enzymes, to undertake chemical transformations of organic chemicals. Biocatalysis has become much more commonplace in recent years, and it is now an important strategy in synthetic chemistry.

BIONEXGEN focused on developing the next generation of biocatalysts tailored to the needs of the chemical producing industries. Biocatalytic process can deliver key advantages over synthetic chemical production methods, including the ability to reduce energy inputs, decreased use of metal-based catalysts, and completely selective access to specific products without unwanted side products. As well as the potential to completely replace some traditional chemical manufacturing processes, biocatalysis can also be used in combination with chemical catalysis and synthesis in order to produce efficient, clean integrated processes for the production of valuable chemical compounds.

BIONEXGEN drew academic expertise from research institutions across Europe: in biotransformations and biocatalysis (The University of Manchester, University of Stuttgart, Academy of Sciences of the Czech Republic, University of Groningen, and University of Oviedo); biotransformations for metabolite synthesis (Leibniz Institute of Plant Biochemistry); production and optimisation of enzymes for industrial use, (Austrian Centre of Industrial Biotechnology); applied biocatalysis for synthesis of fine chemicals and polymers (Royal Institute of Technology, Sweden); enzyme immobilisation (Technical University of Slovakia); and biocatalytic process engineering, including life cycle analysis and economic assessment (Technical University of Denmark and University College London).

Working in collaboration with these leading researchers where innovative industrial partners: including SMEs from the fields of discovery and development of novel pharmaceuticals from natural products and biocatalytic synthesis (EnterChem SL), white biotechnology (GALAB Laboratories GmbH), enzyme immobilisation and green biocatalysis (CLEA Technolgies BV and LentiKat's a.s.); and the world's largest chemical company, with expertise in chemical manufacturing and industrial biocatalysis (BASF SE). Chemistry Innovation Ltd. provided business innovation, knowledge transfer, and dissemination expertise to help the consortium to communicate the aims and results of the project throughout the Industrial Biotechnology community and to the wider European public.

BIONEXGEN has delivered new technologies for diverse applications, including in the biocatalytic manufacture of fine chemicals and pharmaceuticals, bio-based polymers and functional materials; for use by the food and drink industry; and for industrial wastewater treatment to green conventional manufacturing processes. At the same time, the tools for life cycle, environmental, and economic analysis have been developed which will allow direct comparison between bio-processes and their conventional chemical counterparts. By identifying the metrics required for biocatalysis to offer real benefits, it is possible to understand how and when to implement these new technologies to replace or complement existing chemical methods.

PUBLISHABLE SUMMARY Please provide an executive summary. The length of this part cannot exceed 1 page

The aim of **BIONEXGEN** was not only to increase the uptake of the biotechnology at the partner organisations, but also to increase public understanding of industrial biotechnology and green chemistry issues by communicating the scientific programme to the widest possible European audience.

PUBLISHABLE SUMMARY Please provide a summary description of the project context and the main objectives. The length of this part cannot exceed 4 pages.

The **BIONEXGEN** project was conceived as a flagship collaboration to bring together the widest possible expertise in biocatalyst discovery, design and development, including industry leading manufacturers and biocatalysis end-users, together this partnership would *develop the next generation of biocatalysts to be used for eco-efficient manufacturing processes in the chemical industry.*

During inception, the consortium of industrial and academic partners identified the key technology fields of amine synthesis, polymers from renewable resources, glycoscience and wider oxidase application as four key research and development foci based on industry requirements. Within those fields, the project would deliver the next generation of biocatalysts, along with key enabling technologies and know-how to implement processes that will lead to improvements in both economic and environmental performance of the chemical manufacturing industries.

By design, the technologies developed in **BIONEXGEN** would aim to enable industry to use renewable resources with reduced greenhouse gas production compared to their fossil counterparts, and deliver biotechnological routes with reduced energy consumption and less toxic wastes compared to conventional chemical processes. Routes to specialised, high-value chemicals (e.g. chiral chemical compounds) normally require long chemical synthetic routes involving complex reaction steps with toxic side products and waste streams and this project will allow these methods to be replaced by clean biocatalysis routes.

To broaden the range of fine and speciality chemicals and intermediates produced by biotechnological routes, the research addressed: (i) design and optimisation of enzymes to be used in synthetic chemistry; (ii) the selection and development of modified biocatalysts which are resistant to heat, pressure or low pH when used in the production of chemical entities and; (iii) allow the integration of biotechnological steps into conventional chemical processes. The last of these is crucial for the adoption of biocatalysis by the chemical manufacturing industries, who require processes and technologies which can be easily integrating into existing supply and manufacturing chains, with the lowest possible costs to infrastructure. The project would develop and integrate with chemical steps the biotechnological manufacturing routes for the synthesis of fine and speciality chemicals, especially amines, oligosaccharides and renewable polymer intermediates which are better in terms of eco-efficiency, economic potential, complexity and /or specificity of the synthetic pathways than those currently employed. Economic viability and eco-efficiency would be evaluated for key technology platforms, developing metrics which could be assessed on a quantitative basis, guiding process optimisation, performance evaluation, and criteria for critical comparison to existing technologies.

BIONEXGEN was a large and complex collaborative project, and therefore incorporated a fully integrated project structure in order to ensure that the process requirements of end-users were considered from the earliest stage of the project. This integration allowed the efficient translation of research expertise into scalable proof-of-concept experiments. The project was split into 8 research Work Packages (WPs) in order to provide the necessary focus to address the challenges: WPs 1-4 focused on the key technology fields; WPs 5-8 related to the associated enabling technologies.

WP 1: Amine Synthesis. Amines are amongst the most important family of industrial compounds, utilised in bulk for the manufacture of pharmaceuticals, polymers, speciality chemicals and agrochemicals. Biocatalytic routes to this diverse family of molecules offer great potential for the reduction of cost and waste. Many existing syntheses required

deracemisation of racemic products, therefore limiting yields to a maximum 50%, since the unwanted enantiomer is lost. Dynamic kinetic resolution of racemic mixtures and other strategies can increase yields to >50%. Crucially, the use of biocatalytic routes offers greater potential for the direct asymmetric synthesis of chiral amines. The targeted synthesis of single-enantiomer pharmaceuticals is particularly important since the adoption of European guidelines, and those issued by the FDA and other regulatory bodies which require that the adoption of singles enantiomers or racemic mixtures are scientifically justified in terms of safety, efficacy, and risk. Chiral amines are common chemicals in many household applications, such as polyamides used in durable textiles. Work package 1 aimed to optimise and expand the biocatalytic toolkit for chiral amine synthesis, further developing and expanding the substrate range of previously well-characterised enzymes such as Monoamine oxidase (deracemization) and ω-Transaminases (C=O to C-N), and introducing new optimised activities including Phenylalanine Ammonia Lyases (C=C to C-N) and Imine Reductases (carbon-nitrogen double bond reduction). In each case the development of enantiocomplementary enzymologies was targeted in order to allow access to either stereoisomer. The incorporation of mulitenzyme cascades would provide not only successive modifications of the target compounds, leading to complex or previously inaccessible products, but also utilise auxiliary enzymes for the regeneration of the cofactors required for the synthetic enzymes. The use of cascades and cofactor recycling systems would be a key strategy across the WPs to maximise the efficiency and economy of the processes developed.

WP 2: Renewable Resources in Novel Polymer Chemistry. Polymers are the largest volume of chemical products on the market and there is a strong need for bio-based polymers, although the portfolio of such is still limited. In 2010, the global production capacity of bioplastics was estimated at 1000kTon, and this is estimated to rise to >6000kTon by 2017[1]. Starch and cellulose are plant-derived materials, and are used in polymer blends for many applications. Plastics are the biggest consumer of fossil resources other than energy and transport. The growth in demand for bio-plastics has been sharp, however the current offering is very limited, and often to non-biodegradable 'Drop-In' bio-plastics which are identical to fossil-derived products, such as bio-PE and bio-PET. These can replace or be used in combination with conventional fossil-based materials in order to Green products by the use of more sustainable feedstocks. However, growing demand for bio-plastics has placed increased demand on feedstocks, including sugar[2], the growth rate of demand is expected to outstrip that of sugar for biofuel or food production. Improved fermentation technologies (developed in conjunction with WP5) for the production of monomers by microorganisms have the potential to improve yields and maximise productivity. Enzymatic synthesis of novel monomers has the potential to not only efficiently utilise renewable feedstocks, but also to access new polymers with improved properties. This WP aimed to identify new monomers based on renewable materials which can be manufactured efficiently, before using enzymatic synthesis, for example with lipases, to produce polymers with improved properties. Cytochrome P450 and the lipoxygenase enzymes have great potential for the production of monomers from fatty acids, in the form of hydroxylated fatty acids and dicarboxylic acids.

WP 3: Glycoscience and Oligosaccharide Synthesis. This area is amongst the most challenging in organic synthesis, often requiring complex reaction strategies. This WP will develop biocatalytic methods for glycoside and oligosaccharide synthesis and apply them to industrial targets. Polysaccharides, such as starch and cellulose, derived from renewable plant resources can be modified for use as function materials, for example as hypoallergenic

[1] Source: European Bioplastics/Institute for Bioplastics and Biocomposites (December 2013)
[2] Source: *World Bioplastics* (Overview), The Freedonia Group Inc. (November 2013)

superabsorbent hydrogels for use in baby nappies. Modified starches find many diverse applications, including amongst many others, as emulsifiers, rheology modifiers, disintegrants, and surfactants. Many of the conventional production processes rely on the use of inorganic acids, strong alkalis, or other reactive chemical agents, and as such enzymatic modification, for example by sugar oxidases, offers the potential benefits of milder and safer production, along with improved control over the final product and novel functionality. Smaller oligosaccharides are of interest as pre- and probiotics, pharmacueticals, and in biotechnological applications. The benefits of human milk oligosaccharides have been increasingly recognised in the literature, and production of these compounds to improve formula milk is a major goal within the field. Glycosyltransferases are the key enzymes that catalyse the transfer of an activated sugar to complex gylcans, and offer the potential for enzymatic syntheses of oligosaccharides for foods (e.g. sialyllactose, fucosyllactose). One of the key objectives of this WP was to design and develop transferase enzymes that can be applied for the manufacture of these products. Within this WP, the efficient use of sustainable, natural feedstocks was a key requirement for all of the processes developed. Enzymatic modification is a key technology within the food and beverage production industries for example in flavour enhancement. α-L-Rhamnosidase is currently applied in debittering of citrus juices, but also has potential for selective trimming of the readily available glycoside rutin to produce isoquercitrin, a natural product isolated with low yields, but with the potential for production as a high value nutraceutical compound. The optimisation of a biocatalytic process (in conjunction with WP5 and 7) using this enzyme for production of isoquercitrin in kg amounts would be a key demonstration of this technology in industrial manufacturing.

WP 4: Industrial Applications of Oxidases. Oxidase enzymes have huge potential to remove hazardous and environmentally damaging chemical oxidants currently employed across a wide range of processes. This WP will develop efficient oxidative biocatalysts that will be valuable for use in a variety of industrial sectors. Of particular interest are laccases, particularly in combination with the chemical TEMPO mediator system, applied for the application of oxidation of diols or amino alcohols to produce the important building blocks, lactones and lactams. Halogenated compounds are ubiquitous in nature, and have important applications as chiral building blocks and library compounds for the pharmaceutical and agrochemical industries, however they often represent synthetically challenging targets for conventional chemical routes. Haloperoxidase enzymes offer probably the greatest potential for the selective biosynthesis of organohalogen compounds, but were previously underdeveloped for application; few were known, and the production of those enzymes was known to be particularly challenging. In this WP, novel halogenases would be targeted for production and their activities characterised, looking towards future applications in fine chemicals and pharmaceuticals. This WP also developed the use of oxidases for the conversion of phenylpropanoids for the flavour and fragrance industry, a target identified by Symrise flavors AG (the World's 4th largest F&F manufacturer, and a potential route to market for these products). In particular, this WP would target enzymes including Cytochrome P450s methyltransferases, and prenyltransferases, for the synthetic production of chemically inaccessible polyphenol products, where the natural supply is limited by yield or the rarity of the natural producer.

WP5: Fermentation Science. Development of efficient production strains and fermentation techniques for manufacturing enzymes and biocatalysts is critical to economic and process performance. In many cases, the production and preparation of the biocatalyst represents a major contribution to the cost of biocatalytic manufacturing processes. The ability to produce

enzymes in sufficient quantities for application is a significant barrier to translation of interesting laboratory-scale reactions into large-scale industrial processes. WP5 aimed to develop adaptable high-cell density fermentations for multiple host organisms and novel fermentor configurations to improve operational parameters. This required a comprehensive strategy to consider optimisation of existing and new production host microorganisms, host choice, and molecular biological techniques to improve genetic constructs for protein production. All technical partners would contribute to WP5 in order to improve the performance of the enzymes established in WP1-4.

WP6: Biocatalyst Supports and Integration with Chemocatalysts. WP6 examined the role of biocatalyst immobilization as a method to apply biocatalysts to industrial chemical synthesis. Supported enzymes and whole cells have proved to be an invaluable technique for the use of biocatalysts in industrial applications. Immobilisation of biocatalysts on solid supports (such as PVA hydrogel in the form of Lentikats Biocatalyst) or as Crosslinked Enzyme Aggregates (CLEAs) can produce process improvements by stabilising enzymes, simplifying separation and recovery of products, and allowing recycling of the biocatalyst. Laccase is a particularly attractive candidate for improvement by immobilisation, since this broad-activity oxidase has relatively low operational stability due to its ability to oxidase its own surface residues. Additionally, laccase requires chemical mediators (such as the N-oxy radical TEMPO) for the efficient oxidation of many industrially relevant compounds, and immobilised biocatalysts may offer advantages in combined chemo- and biocatalytic systems. Along with laccase, this WP took promising enzymes from WP1-4 for immobilisation and performance evaluation, as single enzymes, immobilised in combination with multiple enzymes, and in combination with cofactor recycling systems.

WP7: Bioprocess and Chemical Engineering. Biocatalysts which perform well under laboratory conditions do not necessarily scale-up to industrial processes, and this performance gap is often exacerbated by the use of existing equipment in order to minimise infrastructure costs. Whilst these technologies, for example stirred tank batch reactors, have proved suitable for simple, robust enzymes, such as lipases and hydrolases, next generation biocatalytic systems may require alternative configurations, in order to incorporate greater demands for cofactors and gaseous coreactants, use of multienzyme cascades, and increasing integration of chemo- and bio- systems. This WP delivered process engineering to implement new biocatalytic processes, by taking selected, relatively mature enzyme systems produced in WP1-4 for evaluation and assessment of their potential for scale-up. Model systems were used for identification of reactor designs, based on performance characteristics such as kinetics, sheer stability, particle size, and oxygen demand. Consideration was given to novel reactor configurations, including *in situ* product removal to alleviate product inhibition of the biocatalyst. Early stage bioprocess design data was generated using automated microscale methods for parallel screening of process conditions.

WP 8: Economic, Environmental and Life Cycle Analysis. Environmental benefits are often claimed for the use of potential biocatalytic processes, versus existing technologies, however prior to this project, surprisingly few specific comparisons had been made of biocatalytic processes and competing technologies. A key objective was to develop simplified methodologies which will enable quantitative assessment via a suite of simple metrics. Economic evaluations of the processes were used to identify bottlenecks and set strategic development targets. WP8 aimed to propose suitable process development propose suitable process development strategies (based on process performance evaluation) for the next generation biocatalysts developed in WPs 1-4. Process improvements would be achieved not

only from process engineering, but also from molecular biology (improved biocatalysts) and chemistry (improved reaction chemistry). For thermodynamically challenged reactions, this approach is particularly relevant, as the process bottlenecks might not originate only from the biocatalytic system (e.g. biocatalyst activity and stability) but also (and often mostly) from the extent of reaction (e.g. reaction yield) and the equilibrium position of the desired process.

WPs 9 and 10 were not research packages, but instead focused on the overall Project Management and Dissemination. The aim of **BIONEXGEN** is not only to increase the uptake of the biotechnology at the partner organisations but also to increase public understanding of industrial biotechnology and green chemistry issues.

WORK PACKAGE 1: *Biocatalytic amine synthesis.*

1. A toolkit for chiral amine synthesis. Over the course of **BIONEXGEN**, a key objective of WP1 was to produce a suite of enzymatic tools for the regio- and enantioselective synthesis of chiral amines from readily available chemical precursors, such as alcohols. Strategies for the efficient enzymatic transformation of alcohols to amines were identified at the start of the project, utilising both relatively mature biocatalyst systems which would require further development for incorporation into processes, but also novel activities known only from the relatively rare examples in literature. Biotransformations were developed using isolated enzymes and whole cells.

1.1 Hydroxynicotine oxidase. A comprehensive set of tools for the manufacture of amines requires enantiocomplementary enzymologies in order to access both stereoisomers. The monoamine oxidase from *Aspergillus niger* (MAO-N) has been an enzyme of considerable interest to UNIMAN, with numerous variants available to oxidise a broad range of substrates. MAO-N generally exhibits (*S*)-amine selectivity, and attempts to identify (*R*)-selective MAO enzymes or to switch selectivity via protein engineering were previously unsuccessful. 6-hydroxy-D-nicotine oxidase (6-HDNO) was identified through a combination of genetic sequence mining and literature searches as a potential candidate. The desired (*R*)-amine selectivity was initially demonstrated using a solid-phase developed for MAO: 6-HDNO was active towards D-nicotine (Figure 1A), but not L-nicotine (1B) or the no-substrate control (1C). Initial characterisation of 6-HDNO demonstrated the limited substrate scope of the wild type enzyme, and therefore mutagenesis was undertaken, targeting the active site of the enzyme in order to broaden the range of accepted substrates. Good conversions were achieved, with excellent enantiomeric excess (e.e.). By this approach, the substrate scope was significantly expanded whilst the selectivity was maintained, thus delivering an enzyme with true enantiocomplementarity to MAO-N.

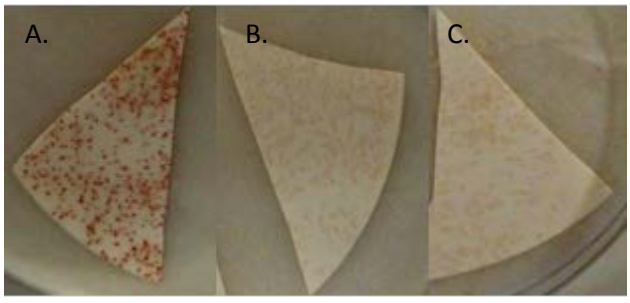


Figure 1: Solid phase screening for 6-HDNO (*R*)-selectivity.

1.2 Imine Reductases. Early in the project, imine reductases were identified as a potential target for use in biocatalytic cascades for the conversion of alcohols to amines. However, few examples were recorded in the literature, and the substrate scope and activity was poorly characterised. (*S*)-imine reductase (*S*-IRED) from *Sterptomyces* sp. GGF3546 was identified in literature as a candidate enzyme for the asymmetric reduction of imines to amines (Figure 2A). The SRED was cloned by UNIMAN and expressed, and the activity of the protein was

characterised towards a broad panel of substrates (2B), generally achieving very high levels of conversion with excellent e.e.

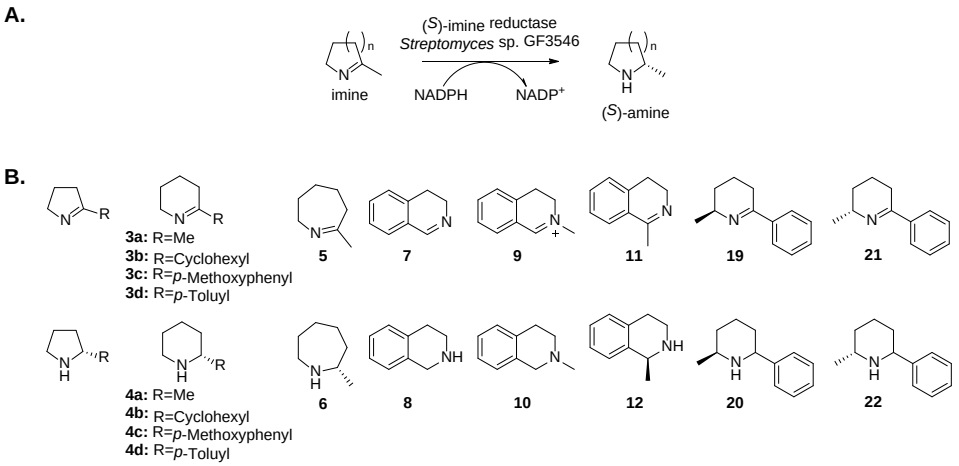


Figure 2: A) Reaction of *S*-IRED from *Sterptomyces* sp. GGF3546; B)Substrate scope (top) and corresponding products (bottom)

An (*R*)-imine reductase (*R*-IRED) from *Streptomyces* sp. was cloned and expressed, which was then characterized towards the same panel of substrates. Despite some differences in the substrate preference and activities of the (*S*)- and (*R*)-IREDs, broadly enantiocomplementary tools were achieved.

Multienzyme cascades for production of chiral amines

Within WP1, a number of different multienzyme cascades were developed towards economical and eco-efficient synthesis of chiral amines.

1.3 Dynamic kinetic resolution using IRED and MOA-N/HDNO. An enzymatic deracemisation system was developed at UNIMAN by coupling whole cell MAO-N or 6-HDNO biocatalyst with the complementary whole cell IRED biocatalyst. Selected biotransformations using 6-HDNO and (*R*)-IRED, with glucose supplementation for cofactor regeneration (exemplified in figure 3A), routinely yielded >99% conversions with >99% e.e.

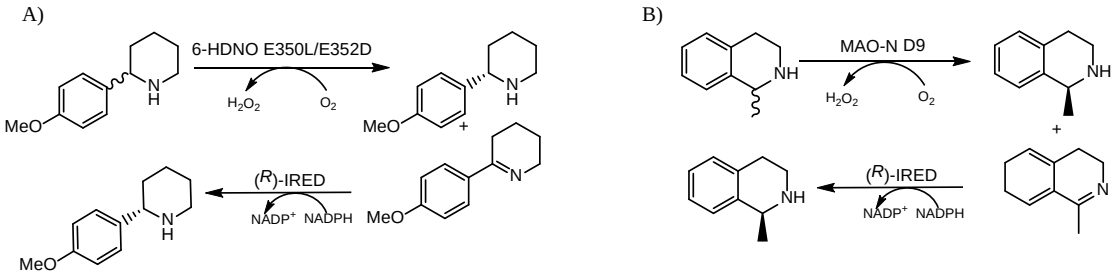


Figure 3: Dynamic resolution using IRED and MAO-N/HDNO

Furthermore, both combinations of genes were coexpressed in the same cells and successfully applied to model biotransformations, although conversions were lower.

1.4 Kinetic resolution and asymmetric amination using Phenylalanine aminomutase.

The phenylalanine aminomutase from *Taxus chinensis* (*Tc*PAM) catalyses the interconversion, via trans-cinnamic acid, of (*S*)-α-phenylalanine and (*R*)-β-phenylalanine (Figure 4). RUG engineered the flexible loop region of an aminomutase, it was possible to enhance the reaction rate for the deamination of (*R*)-β-phenylalanine by 44-fold. The amination of cinnamic acid to yield (*S*)-α-phenylalanine and (*R*)-β-phenylalanine (with high e.e.), also proceeded at enhanced rates.

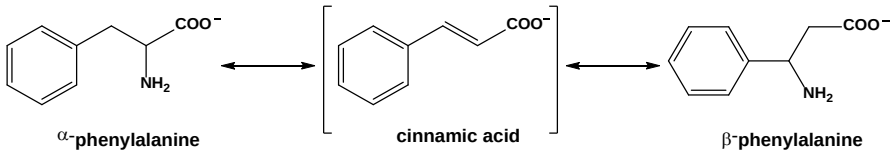


Figure 4: Phenylalanine aminomutase from *Taxus chinensis* (*Tc*PAM) catalyses the interconversion, via trans-cinnamic acid, of (*S*)-α-phenylalanine and (*R*)-β-phenylalanine

Thus, the engineered PAM can be employed both for kinetic resolution of racemic phenylalanine, and also asymmetric amination of cinnamic acid.

1.5 Biocatlytic evaluation of alcohol to amine conversion using a multienzyme approach.

A strategy was devised by BASF with RUG to incorporate the relatively mature enzymologies of ω-transaminases, alanine dehydrogenases, and alcohol dehydrogenases in a biocatalytic cascade to transform alcohols into amines (Figure 5). It is dependent on 3 enzymes: 1) an alcohol dehydrogenase, converting alcohol to the aldehyde; 2) a transaminase for converting the aldehyde to the amine with alanine as the electron donor; 3) an amino acid dehydrogenase, in this case alanine dehydrogenase, converting pyruvate to alanine in a reaction that simultaneously also converts NADH back to NAD^+. This system is the subject of a patent application by BASF and RUG (EP 12183949.2).

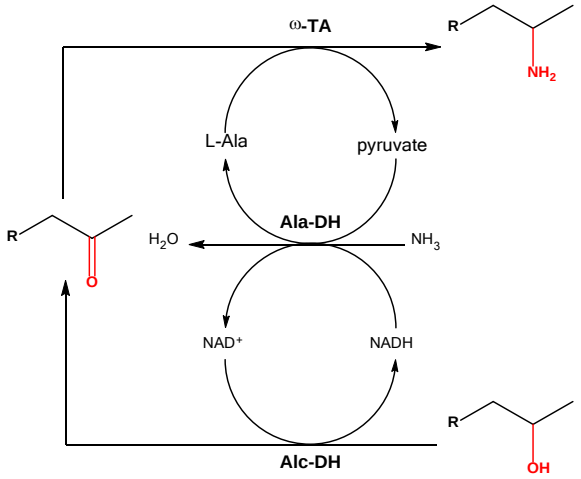


Figure 5: Multienzyme conversion of alcohols to amines

Biocatalytic evaluation of this system was carried out for each enzyme, in order to identify bottlenecks and ensure optimal activity for each step.

1.6 Novel bio-amination cascade.

A novel enzyme system for the biocatalytic cascade synthesis of amines was developed by BASF and UNIMAN during the second reporting period. This system offers considerable potential advantages over other strategies, with simplified components, improved economy, and environmentally benign by-products. The system has so far been demonstrated on ml-scale using two selected industrially relevant alcohol substrates. This system is the subject of an imminent patent application and therefore no details are disclosed in this report.

WORK PACKAGE 2: *Renewable Resources in Novel Polymer Chemistry*

Medium-chain dicarboxylic acids are important monomeric building blocks for the production of various polymers, including high performance polyamides and polyesters. Fats and oils are renewable starting materials, which open up access to next generation biomaterials with improved attributes in terms of performance and eco-efficiency due to their naturally inherent functionalization.

2.1 Cytochrome P450 monooxygenase-catalyzed terminal hydroxylation of fatty acids.

Cytochrome P450 enzymes (CYP) were developed by USTUTT as biocatalysts for the synthesis of industrially relevant ω-oxyfunctionalized medium- and long-chain length aliphatic compounds. Prokaryotic CYP153A enzymes were selected on the basis of their high ω-regiospecificity (functionalizing the terminal carbon), expression in soluble form and application within easily manipulated bacterial systems with good handling characteristics.

The monooxygenases were first screened towards C_5-C_{12} chain-length alkanes and C_6-C_{12} primary alcohols. CYP153A, originally from *Marinobacter aquaeolei* VT8 but produced recombinantly in *E. coli*, was found to oxidize alkanes to primary alcohols and these further to α,ω-diols (Figure 6). Our substrate screening studies towards C_8-C_{20} saturated and 9(*Z*)/(*E*)-$C_{14:1}$-$C_{18:1}$ monounsaturated fatty acids provided further insights into the capabilities of CYP153A enzymes.

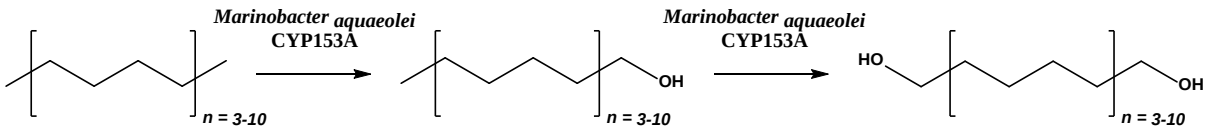


Figure 6: Oxidation of medium chain alkanes to diols using *Marinobacter aquaeolei* VT8CYP153A enzyme.

We demonstrated that the primary alcohol ω-hydroxylase CYP153A *M. aq.* was also able to convert fatty acids to ω-hydroxy fatty acids. CYP153A *M. aq.* exhibited higher flexibility towards fatty acids of different size and saturation level. A rational design approach, based on structural information about the enzyme, enabled us to identify amino acids key for the

activity and selectivity of CYP153A *M. aq*. On the basis of this, a library of variants was created with improved properties for a broader range of reactions. An important aim was achieved by the creation of variant G307A, which was able to ω-oxygenate shorter fatty acids, owing to a higher catalytic efficiency compared to the wild type enzyme. So far, engineered CYPs from other bacterial families are yet not able to compete against CYP153A enzymes in terms of selectivity.

CYP activity relies on efficient coupling of the enzyme with a reductase electron transfer partner. Product yields achieved by *in vitro* reactions used the non-natural CamA-CamB-CYP153A electron transfer chain. Even though CamA/CamB are known to interact reasonably well with CYP153A enzymes, higher oxidation activities should be expected by using physiological redox partners or an artificial reductase in a single polypeptide chain arrangement. The limitations of the *in vitro* system were tackled by producing a protein fusion construct comprised of the CYP153A monooxygenase and the reductase domain of CYP102A1 (P450 BM3). This fusion protein is "catalytically self-sufficient", since it does not require a reductase partner protein. Hydroxylation reactions with the optimized fusion variant CYP153A *M. aq*.(G307)-CPR_{BM3} were applied in a first bioprocess step in a small scale (1L) reactor to investigate biotransformation parameters and synthesize ω-hydroxylated dodecanoic acid. The successful production of the industrially relevant ω-hydroxylated dodecanoic acid at g/L scale (4.0 g/L) in *E. coli* was reported in this project in collaboration with Work Package 5. This is an important demonstration of a new biocatalytic pathway based on renewable substrates, which has the potential to displace classical petrochemical routes to the same products.

2.2 Biocatalytic production of medium-chain dicarboxylic acids.

Dicarboxylic acids are important building blocks and chemical products in their own right. We focused on the production of the dicarboxylic acid azelaic acid, an industrially relevant C_9 building block, used in the manufacture of lubricants and plasticizers, and as bactericide in cosmetic anti-acne treatments. Conventional production of azelic acid is by ozonolysis of oleic acid, a hazardous process involving the use of toxic ozone gas, with the potential to form explosive by-products.

In the biocatalytic process developed by USTUTT in BIONEXGEN, azelaic acid was generated by the oxidative cleavage of the C_{18} long chain fatty acid linoleic acid. The cascade composes of an enzymatic cascade comprising of three different enzymatic reactions (Figure 7**)**. In a first step linoleic acid is transformed into an easily cleavable species (9*S*-HPODE) via a hydroperoxidation reaction catalyzed by the enzyme 9-lipoxygenase (LOX). Introduction of oxygen at position C9 enabled cleavage into two C_9 fragments. The cleavage reaction was performed via a 9/13-hydroperoxid lyase (HPL) and yields 3*Z*-nonenal**,** but also 9-oxononanoic acid, the precursor for azelaic acid. In a last step, 9-oxononanoic acid was oxidized to azelaic acid by an aldehyde dehydrogenase enzyme (ALDH).

Initial proof-of-principle work demonstrated that 9/13-HPLs were *S*-selective on 9-HPODE and that the *in vitro* coupled LOX1 and 9/13-HPL reaction showed significantly better performance when carried out in a successive rather than a simultaneous manner. Using a 9/13HPL 9-oxononaoic acid 76 % yields of 9-oxononanoic acid were obtained, with linoleic acid conversions of 87 %. Detailed investigation of the LOX activation behaviour revealed that in a simultaneous approach the lipoxygenase and the hydroperoxide lyase were in competition for the common reaction intermediate, the hydroperoxy fatty acid. Reducing the HPL activity consequently allowed full LOX activation and enabled the cascade to be performed in a simultaneous manner.

With the aim to transfer the whole enzymatic cascade into an *in vivo* whole cell system the expression levels of LOX and HPL were adjusted accordingly by modifying a standard expression system to produce a dual expression system for simultaneous production of both enzymes. In contrast to the *in vitro* system the established *in vivo* system tolerated high concentrations of substrate, and was furthermore able to catalyze the last step in the enzymatic cascade without purification of the intermediate. The *E. coli* strain developed was able to produce 29 mg/L azelaic acid in 8 h with a selectivity of 47 %. This was an important step towards a viable technology for the industrial bioproduction of azelaic acid.

2.3 Biocatalytic production of medium chain fatty acids and corresponding ω-hydroxylated derivatives.

A wide variety of today's industrial products is based on the group of medium-chain fatty

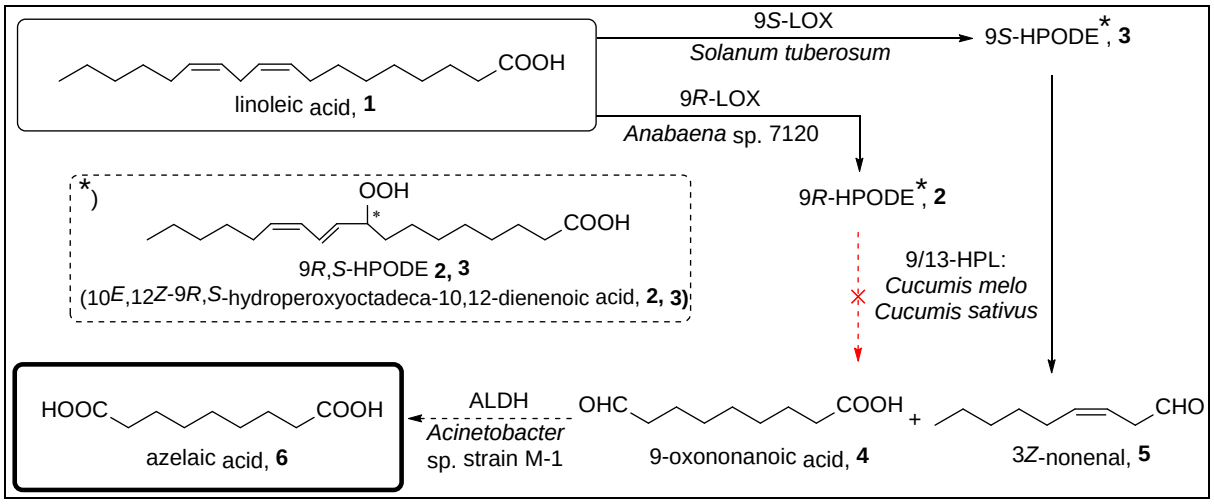


Figure 7: Multienzymatic biocatalytic cascade towards azelaic acid.

acids (6 – 12 carbon atoms) and their functionalized derivatives. Fatty acids are used in lubricants, plasticizers, cosmetics, dyes, food supplements and for medical treatment, whereas their hydroxylated derivatives like ω-hydroxy fatty acids are used directly for the production of polymers, or as flavour compounds and fragrances in their lactonized form. However, the current methods for production of medium-chain fatty acids are either very energy-consuming due to the use of high temperatures and pressures (e.g. hydroformylation

process) or use harmful catalysts like triethylaluminium (e.g. Ziegler process). Owing to these drawbacks, the aim of this project was to develop and establish an alternative production route to medium-chain fatty acids and the corresponding ω-hydroxylated derivatives that is economically and environmentally more sustainable. The strategy selected by USTUTT for this purpose, was engineering *de novo* fatty acid synthesis by the model organism *Escherichia coli*, using the tools of molecular biology.

In this approach, the acyl-CoA synthetase (fadD), which catalyses the first step in β-oxidation (the breakdown of fatty acids), was deleted to allow the general accumulation of fatty acids within the host cell. In order to deregulate the fatty acid synthesis cycle and to shift its product spectrum towards medium-chain fatty acids, the thioesterase FatB2 from the seed of the cigar-plant *Cuphea hookeriana* was overexpressed. This enzyme had been previously described in literature as important for the accumulation of C_8-C_{10} chain length fatty acids. These genetic modifications allowed the *E. coli* cells to accumulate fatty acids of the desired chain lengths. Overexpression of the thioesterase FatB2 in the β-oxidation deficient *E. coli* strain allowed accumulation of the C_8 chain length octanoic acid. Subsequent optimization of various production parameters, such as culture density at induction, inducer concentration, incubation temperature, growth medium, and starting pH, successfully increased the amount of extractable octanoic acid and decanoic acid from 1% to about 70% and 12 % of all measured fatty acids. This represents 620 mg octanoic and 101 mg decanoic acid per litre of medium during 24 h fermentation in a bioreactor.

In the final step, the medium-chain fatty acids produced were hydroxylated by the overexpression of the Cytochrome P450-reductase fusion construct CYP153A *M. aq.*(G307)-CPR_{BM3} (described above) which exhibits ω-hydroxylation activity towards octanoic acid. As a proof of concept, we further successfully established two-cell and single-cell fed batch systems for the production of 8-hydroxyoctanoic acid by the co-expression of the self-sufficient monooxygenase fusion construct and the thioesterase FatB2, finally yielding about 40 mg of 8-hydroxyoctanoic acid in 24 h in a two cell shake flask system. Although these are modest yields, they represent a good starting point for the development of this technology as an eco-efficient method for production of these industrially important compounds.

2.4 Enzymatic routes toward bio-based functional polymers and cross-linked materials.

Enzymes have proved to be powerful and selective catalysts in the syntheses of polymers e.g. polyesters. Polyamides are polymers with improved thermal and mechanical properties but enzymatic synthesis becomes more challenging. Poly(ester-amide)s are polymers with combined properties from polyesters (degradability) and polyamides (strength) in one polymer. Using renewable starting materials generated through process similar to those described above, enzymatic synthesis of functional poly(ester-amide)s and polyamides was achieved by KTH using the enzyme *Candida antarctica* lipase B (CalB) as a robust and efficient catalyst. The enzymatic syntheses were performed in bulk or in an initial amount of solvent at 70-95 °C.

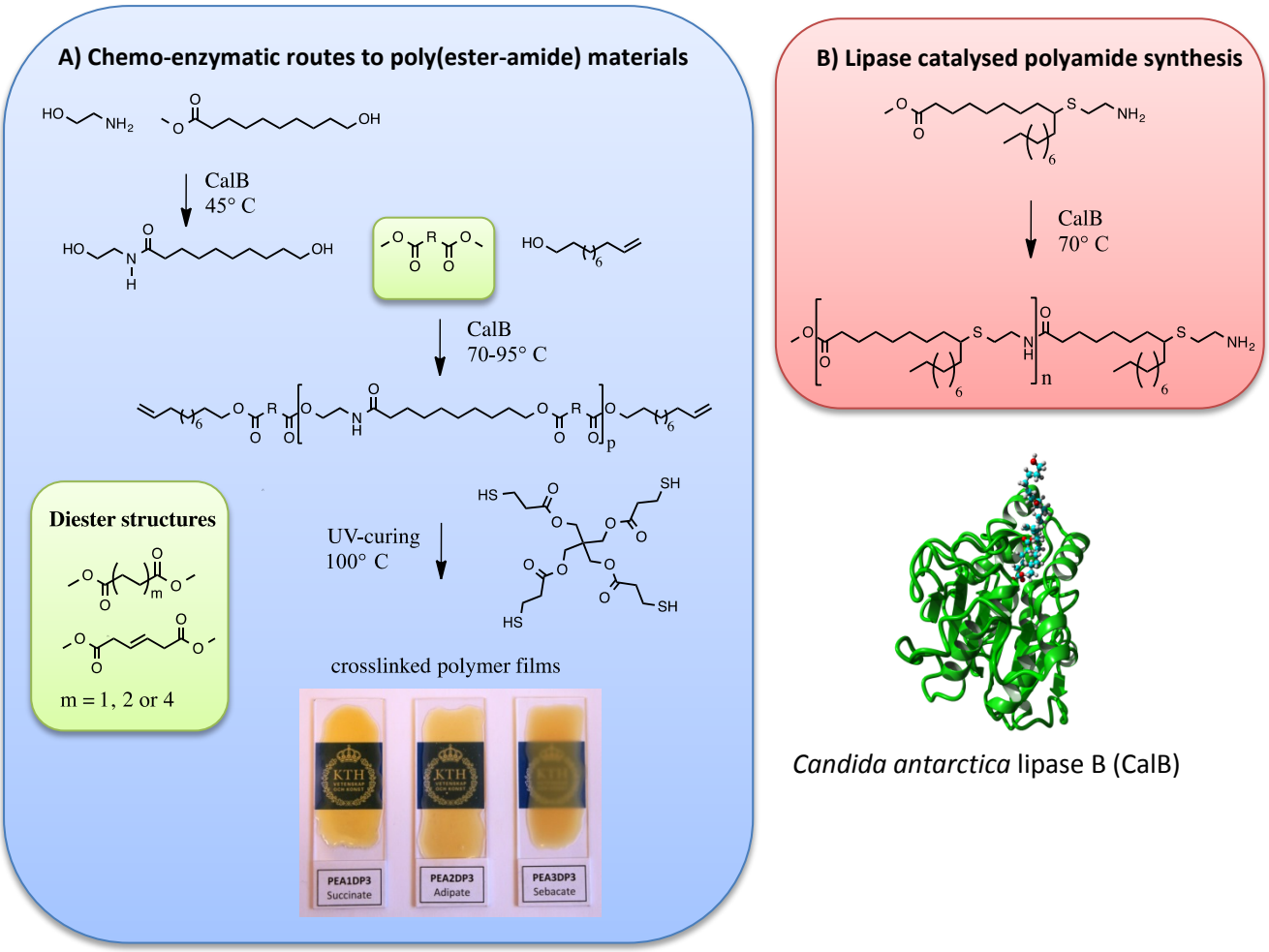


Figure 8: Enzymatic routes toward bio-based functional polymers and cross-linked materials.

Short alkene functional poly(ester-amide)s (macromonomers) were synthesized using lipase catalysis in a two-step procedure (Figure 8A). The alkene functionalities in the macromonomers presented a straightforward route for cross-linking to high conversion by photo-initiated thiol-ene chemistry. The material properties, including functionality, crystallinity, hydrophobicity and melting temperature, could be tailored by the choice of diester (green in Figure 8A) used in the polymer. By selecting the density of thiol-ene cross-links during film formation, material properties could also be altered and unreacted alkene groups can be utilized in a second functionalization step. Derivatives of unsaturated fatty acid esters have been reported as new monomers in polyamide synthesis. Lipase catalysed synthesis of polyamides based on methyl oleate, was achieved with high monomer conversion under mild conditions, 70 °C (Figure 8B).

CalB variants with improved amide hydrolysis specificity were known, and by protein engineering variants with improved amide synthesis performance were developed by KTH, but the enhancement was much smaller than was observed for the use of those variants in hydrolysis reactions. Studies are currently ongoing for further improvements. Lipase catalysis

of the type developed in BIONEXGEN will be a valuable tool in the syntheses of functional polyamides with tailored properties.

WORK PACKAGE 3: *Application of enzymes to gylcoscience and oligosaccharide synthesis*

3. Developing enzymes for oligosaccharide synthesis.

Oligosaccharide synthesis is amongst the most challenging in organic synthesis, often requiring complex reaction strategies to overcome the challenges of producing precise structures from building blocks with numerous functionalities available for reaction. Unlike the other famous natural biological polymers, such as DNA and proteins, there are no simple genetic templates available to direct the sequential addition of monomers to the growing molecule. BIONEXGEN aimed to develop biocatalytic methods by creating highly selective and controllable tools for glycoside and oligosaccharide synthesis and modification, and applications towards industrial targets. Polysaccharides, such as starch and cellulose, derived from renewable plant sources can be modified for use as function materials, for example as hypoallergenic superabsorbent hydrogels for use in baby nappies. Modified starches find many diverse applications, including as emulsifiers, rheology (viscosity) modifiers, disintegrants, and surfactants. Many of the conventional production processes rely on the use of inorganic acids, strong alkalis, or other reactive chemical agents, and as such enzymatic modification, for example by sugar oxidases, offers the potential benefits of milder and safer production, along with improved control over the final product and novel functionality. Smaller oligosaccharides are of interest as pre- and probiotics, pharmacueticals, and in biotechnological applications. Glyco-modifcation of proteins is often key to their role and function, and tools study this natural phenomenon, as well as to mimic natural glycosylation for the production of drug molecules, are amongst the leding challenges in glycoscience. The benefits of human milk oligosaccharides (HMOs) have been increasingly recognised in the literature, and production of these compounds to improve formula milk is a major goal within the field. Biocatalytic production of food oligosaccharide (including HMOs) and glyco-pharmaceuticals requires highly selective enzymes produced in organisms which are recognised as not presenting a risk to human health.

3.1 Novel enzymes for synthesis of oligosaccharides.

During the early project, a literature review revealed a wide range of glycosyltransferases with the potential for application in industrially relevant processes identified by BASF. These enzymes can be used in oligosaccharide synthesis to replace polluting chemical processes, such as acid hydrolysis of large oligosaccharides. Amongst the target enzymes sialyltransferases, cellulases, endo-cellulase, xyloglucanase, *N*-acetylsaminyltransferase, and cyclodextrin glucosyltransferase are able to synthesise a wide variety of oligosaccharides starting from simple building blocks. Glycosyltransferase genes were made available to BIONEXGEN partners, and existing expression technology for the production of respective enzymes was transferred to GALAB.

3.2 Synthesis of oligosaccharides using starch as a renewable starting material.

Starch is an abundant polysaccharide with limited industrial applications due to its physicochemical properties (low thermal decomposition, syneresis, low water solubility and low stability at low pH). However, UNIMAN demonstrated that cyclodextrin glucanotransferase (CGTase) from *Bacillus macerans* was able to carry out the formation of α-(1,4) linked cyclomaltodextrins (CD), which contain between six and eight glucose units, using starch as starting material. Additionally, CGTase performs intramolecular transglycosylation reactions involving the CD and various acceptors carbohydrates, such as alkyl glycosides. The capacity of the CGTase to convert mono-substituted β-cyclodextrins was explored. Also, the transferase reaction of glucose moieties to the several alkyl glycosides (N-dodecyl-beta-D-maltoside (DM) and 2-cyclohexylethyl beta-D-maltoside (CHM)) was studied (Scheme 4).

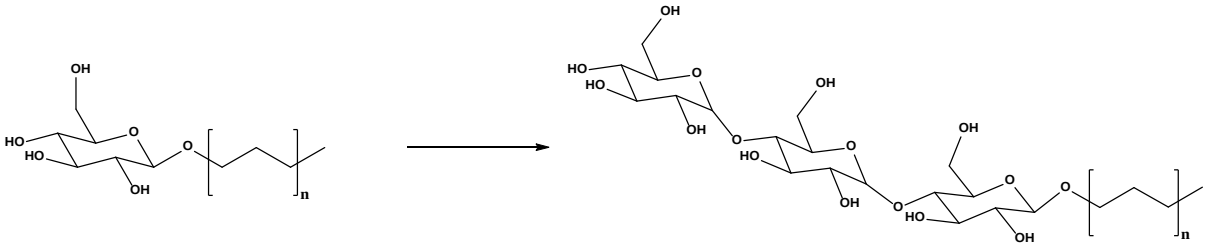


Figure 9: Modification of alkyl glycosides.

3.3 Modification of natural polysaccharides to improve properties.

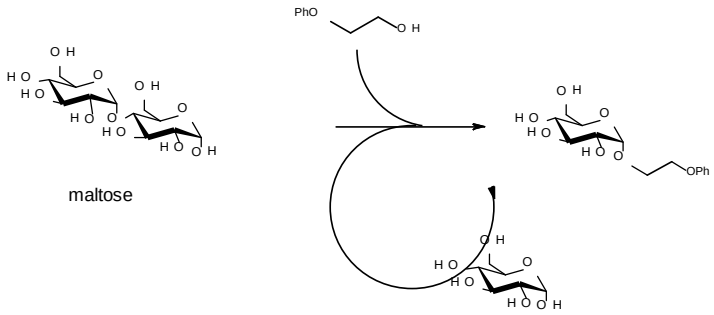
The galactose oxidase (GOase, E.C 1.1.3.9) from *Fusarium sp.* is a monomeric copper-containing enzyme that catalyses the oxidation of the 6-hydroxyl group of galactose. Directed evolution techniques such as Combinatorial Active Site Testing (CASTing) were carried out by UNIMAN in order to increase the activity of the galactose oxidase enzyme for a substrate of interest. Several variants were developed with increased glucose oxidation activity compared to the parental enzyme. However, the yields of soluble enzymes were poor, preventing process evaluation and the development of up-scaled reactions using this biocatalyst. The production of this biocatalyst was improved in collaboration with ACIB (WP5) using two different expression host systems: methanol induction in the yeast *P. pastoris*, and auto-induction in the bacterial host *E. coli*. The ability to produce gram quantities of galactose oxidase was allowed demonstration of oxidations using industrially relevant substrates such as glucosides and lactose.

3.4 Transglycosylases.

Transglycosylases were identified and evaluated for use in industrial applications by BASF. This resulted in identification of cellulase from *T. reesi*, α-amylases from *T. maritime* and *X. campestris*. Cellulase from *T. reesi* was used to transglycosylate xyloglucan polysaccharide using acceptors like propargyl alcohol, *n*-butanol and 4-hydroxy butylacrylate (4-HBR). It was found that 4-HBR is an acceptor for an enzyme, however under optimised conditions, the

hydrolysis/transglycosylation ratio was 6:1. Additionally, no activity was detected towards poly-(ethylene)propylene glycols.

Glycosylation of different petrochemical alcohols was achieved using α-amylases from *T. maritime* and *X. campestris* (XgtA). These were applied at pilot scale in order to provide material for further process and product evaluation. For example, glucosides of *n*-butanol and phenoxyethanol were prepared on a gram scale with XgtA (Figure 10). Furthermore, we found that XgtA possesses a very broad acceptor scope, with activity towards phenolic, benzylic and aliphatic alcohols.



Figrue 10: Transglycosylation of maltose for synthesis of phenoxyethanol gucoside.

3.5 Gylcosyltransferases.

Glycosyltransferases were developed by GALAB for the synthesis of sialyllactose and fucosyllactose, the most abundant oligosaccharides in human milk. The enzymes involved in the formation of these important HMOs are sialyltransferases and fucosyltransferases respectively. Two biocatalytic approaches for the synthesis were followed: using isolated enzymes or whole cell biocatalysts. For the *in vitro* approach, *E. coli* systems were optimised for high yield production of glycosyltransferases.

Within BIONEXGEN, a number of novel glycosyltransferases were successfully produced: α-2,6-sialyltransferase from *Photobacterium damselae*; β-2,3-sialyltransferase from *Neisseria Meningitis*; 1,2-Fucosyltransferase from *Heliobactger Pylori*; 1,2-Fucosyltransferase from *Colwellia psychererythraea*; and 1,2-Fucosyltransferase from *Thermosynechococcus elongates.* Oligosaccharide synthesis was demonstrated with 1,2-FucTs, and all techniques for the *in vitro* syntheses of mg amounts of oligosaccharides successfully established. The limitation of this reaction is the availability of the donor sugar nucleotide. GDP-fucose as well as CMP-sialic acid is very expensive and only available via chemical synthesis in larger quantities. Therefore, we followed a whole cell approach using pathway engineering to programme the cell for the target reaction.

Saccharomyces cerevisae was chosen by GALAB as host organism for the biocatalysis of fucosyllactose. The organism is generally recognised as safe (GRAS) which is important in food production. Furthermore, the cells own salvage pathways required that fewer recombinant enzymes were introduced into the cells. However, *S. cerevisae* is not able to transport lactose though the cell membrane, and therefore a lactose permease was introduced

to facilitate the uptake of the disaccharide by the cell. By this method, a pathway to form GDP-fucose in *S. cerevisiae* using a two-step enzymatic reaction starting from fucose via beta-L-fucose-1-phosphate was successfully established. In an alternative reaction, the enzymes GMD and wcaG were introduced, and starting from GDP-mannose which naturally occurs in the cell, GDP-L-fucose was formed in a two-step, two-enzyme reaction.

Within BIONEXGEN several active glycosyltransferases were successfully developed, and used for proof-of-principle demonstrations for production of oligosaccharides. For large scale cost-effective production it is necessary to find strategies to include the sugar nucleotide production. This can be a whole cell approach or the multistep enzymatic approach, and the tools described above offer one such route. BIONEXGEN has contributed promising new techniques in synthetic biology which open new horizons in complex multistep enzymatic reactions.

3.6 α-L-Rhamnosidase for selective trimming of rutin.

α-L-Rhamnosidase is currently applied in debittering of citrus juices, but also has potential for selective trimming of the readily available glycoside rutin to produce isoquercitrin, a natural product isolated with low yields, but with the potential for production as a high value nutraceutical compound.

α-L-Rhamnosidase from *Aspergillus terreus* was chosen based on a large screening effort of fungal producers (approximately 100 strains) carried out at IMIC. This enzyme was produced extracellularly and in sufficient amounts for further evaluation. This enzyme was found to be thermo- and alkali-tolerant, and was able to operate at 70°C and pH 8.0, allowing its use for derhamnosylation of many natural compounds (e.g., rutin). However, *Aspergillus terreus* also produced extracellular β-D-glucosidase, which was undesirable for the conversion of rutin: β-D-Glucosidase would cleave the glucose moiety from the rutin bioconversion product, isoquercitrin (Figure 11), to yield the unwanted compound quercetin.

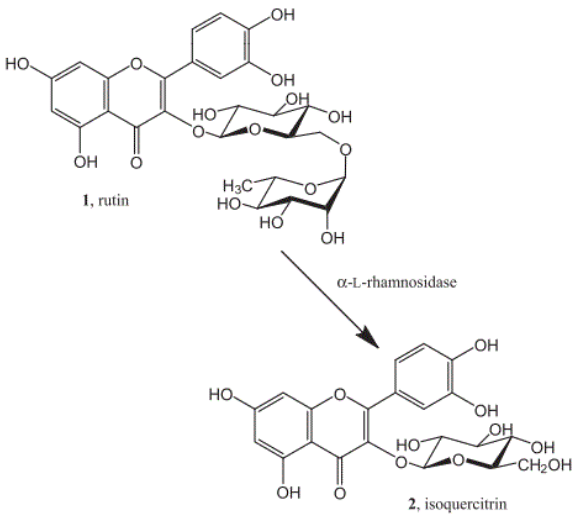


Figure 11: Selective trimming of rutin by α-L-Rhamnosidase to produce isoquercitrin.

The recombinant extracellular *Aspergillus terreus* α-L-Rhamnosidase was produced in the yeast *Pichia pastoris*. The effective overexpression was optimized, and eventually the recombinant α-L-Rhamnosidase was produced as extracellular protein with three times higher activity and in a half time compared with the natural producer. Additionally, compared with the native enzyme, the recombinant version showed higher activity in the alkaline region (up to pH 11.5), which is very important for the solubility of the flavonoids used as substrates. The recombinant enzyme showed an extremely broad and rather flat pH profile (1.5 – 11.5), which is a major advantage for biotechnological applications, and was stable at temperatures up to 80°C.
Recombinant α-L-Rhamnosidase production was optimised. Commercial and very expensive YNB medium was replaced by a cheaper salt solution developed during media optimisation experiments. This was a fundamental step towards economically effective enzyme production, since the cost of growth media was a significant barrier to commercialisation of this enzyme. Furthermore, the use of this defined media considerably simplified purification of the secreted protein product from the culture medium.

Immobilisation studies were conducted in collaboration with WP 8. The enzyme was immobilised in PVA (polyvinylalcohol) gel capsules - LentiKats® with activity 7 Ug^{-1} giving 21% of its original activity. Although this was a significant decrease in activity, this was not unexpected, whereas by encapsulation in LentiKats®, the enzyme could easily be reused and the immobilised enzyme retained activity throughout repeated cycles of use. Immobilisation did not significantly affect the pH and temperature profiles of α-L-rhamnosidase, K_M increased 3.4 times whereas V_{max} decreased more than 10 times.

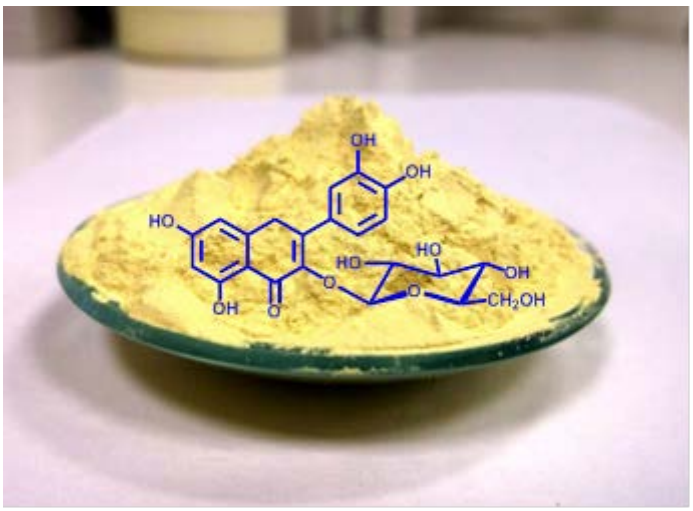


Figure 12: Final product isoquercitrin

The procedure for isoquercitrin production by α-L-rhamnosidase from *Aspergillus terreus* (recombinant) has been demonstrated and verified at 21L scale. Using a rutin substrate concentration of 100 g/L (2 Kg in total), bioconversion proceeded in 17 hours at 70°C, pH 8.0: conversion was 97.5%, with a final yield after processing of 87% of the theoretical maximum, in total, 1.235kg of isoquercitrin was recovered (Figure 12). During this large-scale demonstration experiment we could also develop and verify final product processing

methodologies. BIONEXGEN allowed development of a large scale process for the production of isoquercitrin, and also delivered sufficient quantities to allow product evaluation and testing for commercial applications.

WORK PACKAGE 4: *Industrial Application of Oxidases*

Biological systems for redox processes using mild reaction conditions have been gaining significant relevance in recent years, with huge potential to remove environmentally damaging chemical oxidants and reductants. In particular, oxidases are emerging as one of the most remarkable classes of biocatalysts for the synthesis of pharmaceuticals and high added value compounds. Their potential to catalyze oxidative processes under mild and benign reaction conditions, have allowed their implementation for synthetic purposes in industrial manufacturing.

This work package targeted identification of robust biocatalyst, aiming to later improve their activity and stability by means of immobilization processes or directed evolution techniques, and optimise those parameters that affect selected biotransformations with high potential for application at industrial level. Work Package 4 has delivered significant advances in this area, providing the market with a number of very different classes of redox enzymes, such as laccases, oxidases, halogenases, O-methyltransferases and aromatic prenyltransferases.

A key objective WP4 was to identify and develop robust oxidases with characteristics that have been identified as useful for industrial applications. Therefore CLEA Technologies BV worked on the immobilization of several oxidases in the form of Cross-linked Enzyme Aggregates (CLEA) and made them available for the other partners within this WP and others. CLEAs are produced via, firstly, aggregation of the enzyme from an aqueous environment, followed by cross-linkage to form immobilized enzyme particles (Figure 13).

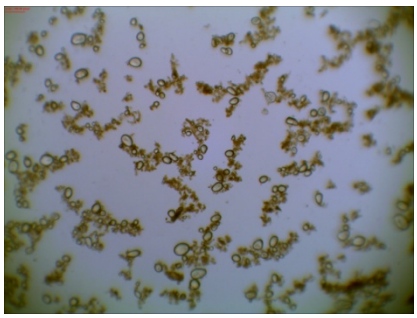


Figure 13: Microscopic images (10x) of Laccase CLEA particles.

4.1 Glucose oxidase/catalase combi-CLEA.

A glucose oxidase (GOx) CLEA and a combi-CLEA of GOx and catalase was prepared and made available for the other partners. For this two-in-one catalyst, a so called *combi-CLEA*, a one-step immobilization was efficiently performed for two enzymes. This method is economically more viable since it reduces the production steps and employs a single precipitant and a crosslinker for the immobilization. Moreover, it has also the benefit that one

enzyme can act as a proteic feeder for the second enzyme. This can result in decreased diffusion limits within the CLEA. The combi-CLEA shows higher activity recoveries than the activity recoveries of the corresponding 'single'-CLEAs. The GOx CLEA was tested by DTU in WP7.

4.2 Laccase CLEA.

For this purpose, a variety of commercial laccases were sourced and screened first for enzymatic activity, then for their ability to perform aerobic oxidation of alcohols. Four biocatalysts namely, laccases from *Myceliophthora thermophilia*, *Trametes* sp., *Trametes versicolor*, and *Cerrena unicolor* were identified from this screening programme. The viability of these enzymes to efficiently perform aerobic oxidation was confirmed, yielding high conversion within 4-8 hours. Optimal reaction conditions were also established. Laccase CLEAs from *Trametes* sp. and *T. versicolor* were successfully employed in diol oxidation reactions in combination with an N-oxy radical mediator by UNIOVI. Again in collaboration between CLEA and UNIOVI, environmental and economic assessment of the chemical and enzymatic approaches for lactone synthesis from diols was carried out, demonstrating the excellent potential of oxidases produced in-house.

IPB also assessed these laccases and their CLEAs in screening towards a variety of classes of compounds, including coumarins, flavonoids, anthraquinones and indoles, and the identification of respective products. Application of laccase CLEAs in polyalcohol oxidations and textile waste water decolourization was also performed (see WP6 for details).

The main objective for UNIOVI was the optimization of reaction conditions, using both commercially available enzymes and other enzymatic preparations provided by partners in the BIONEXGEN project including CLEA, LK, and ENTR. This work focussed on two areas; the oxidation of alcohols for the production of carbonylic compounds, and challenging halogenations of aromatic compounds using halogenases.

4.3 Oxidation of diols and amino alcohols.

UNIOVI have developed methodologies for the oxidation of alcohols using a catalytic system based on the combined action of a laccase and TEMPO, an N-oxy radical mediator. The application of this system has provided easy access to industrially relevant, high added value compounds such as lactones or lactams. The efficiency of the catalytic system in both aqueous and also biphasic systems was demonstrated, where limitations related to the substrate solubility were satisfyingly overcome. The *Trametes versicolor*/TEMPO system has been demonstrated as a remarkable biocatalyst for applications in the transformation of diols into lactones with excellent levels of activities and complete conversions (Figure 14). Oxidations occurred in a non-stereoselective fashion, but with complete regio- and/or monoselectivity, obtaining lactones with excellent purity after a simple extraction protocol. This enabled the production of the target products with excellent isolated yields. This catalytic system has been demonstrated to be scalable, compatible with a variety of functionalities, and also allowing successful enzyme recycling using a laccase CLEA preparation.

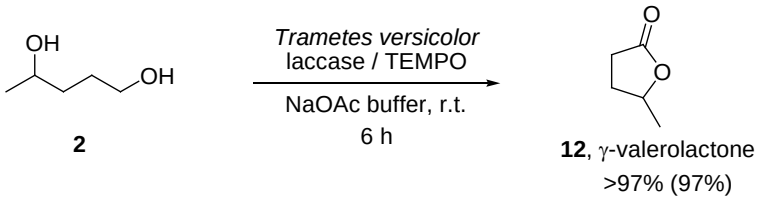


Figure 14: Oxidation of 1,4 pentanediol by the Trametes versicolor laccase/TEMPO catalytic system to yield γ-valerolactone.

The simplicity of the reaction set-up is notable; the oxidative processes were conducted under mild conditions and open to air, thereby conforming to the 12 principles of Green Chemistry. Environmental assessment of the laccase/TEMPO system has been studied through EATOS calculations to compare this method with other traditional chemical approaches. Significant advantages in terms of the E-factor were demonstrated, especially in comparison with metal-catalyzed reactions, since there was no requirement to use expensive and highly toxic reagents.

This methodology has shown to be selective for oxidation of amino alcohols, favouring selective oxidation of amino groups in the presence of hydroxyl groups when such amino groups were not protected. In contrast, by forming the corresponding protected amino alcohols, the reaction was driven towards the hydroxyl group leding to the formation of interesting hemiaminal and enamines in good isolated yields from preparative scale reactions. The preparation of lactams was challenging, but by tuning the protecting group it was possible to detect formation in some cases.

4.4 Tryptophan halogenases.

UNIOVI in collaboration with ENTR have developed systems for halogenation of organic compounds, producing halogenases with potential applications in chemical synthesis. Once the enzymes were isolated, cloned and purified, preparative biotransformations were carried out using tryptophan as a model substrate, obtaining halogenated aromatic compounds with complementary regioselectivities depending of the halogenase source (Figure 15).

The main objective for ENTR was focused on two distinct technology areas. On one hand, and during the first 18 months of the project, efforts were made in the overexpression of enzymes, mainly oxygenases, to be used by the partners of BIONEXGEN. In the second part of the project, the production of halogenases with suitable synthetic properties was undertaken.

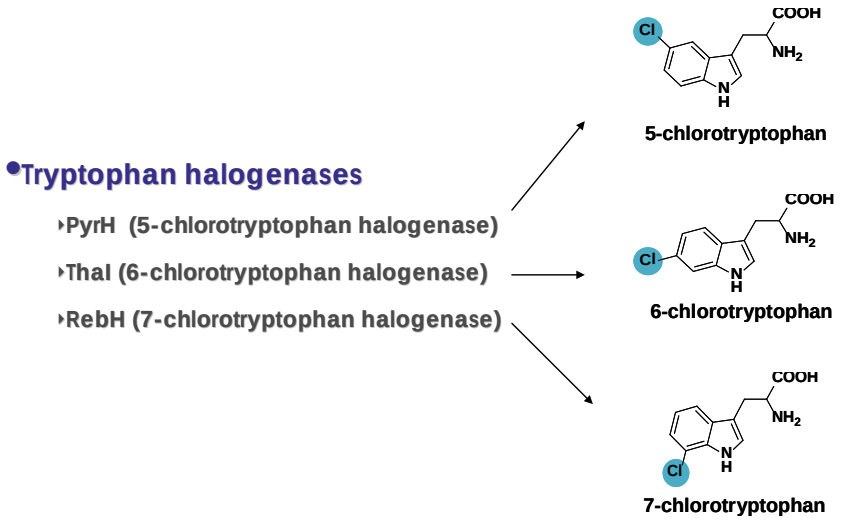


Figure 15: A suite of tryptophan halogenases for regioselective halogenation of tryptophan.

ENTR focused in the production and testing of different halogenases for their ability to produce chlorinated compounds using tryptophan as a model substrate, but also other non-natural substrates were screened as well.

Halogenases encoding genes RebH, ThaI and PyrH were cloned into expression vectors for the production of the enzymes. Initially, work concentrated on the production of His-tagged proteins, as a well-established method for simple purification of recombinant proteins. ENTR was able to overexpress in *E. coli* all of the His-tagged enzymes initially proposed. Cell-free extracts of halogenases and flavine reductase were prepared from *E. coli* and transferred to UNIOVI to carry out specificity assays. Towards the end of the project, a literature report was published by the group of Payne, *et al.* (2013) describing an alternative strategy using chaperons and maltose-binding protein (MBP) instead of a His-tag. Their reported strategy apparently achieved good results towards the same targets. Therefore ENTR undertook to repeat that work: halogenases were co-expressed with a chaperone system in order to improve their solubility, and the flavin reductase was fused with the maltose binding protein (MBP) for improved solubility. This approach was unsuccessful and the reductase, RebF, alone was obtained in abundant soluble yields.

Activity assays were performed using the halogenases expressed earlier as His-tag fusion proteins, and activity was detected, only towards the natural substrate tryptophan, but not other, synthetic substrates. A set of tryptophan-related compounds was tested in halogenation assays with the RebF flavin reductase and the different halogenases. The most successful combination coupled RebF with RebH halogenase. The reaction was found to be sensitive to oxygen concentration (partial pressure), however this parameter is difficult to control at small scales. Chlorination studies were also performed with the chaperone co-expression system with the different halogenases. RebH co-expressed with Gro7 obtained bioconversion ratios of approximately 40%., and scale up was explored to 100mg scale, but with lower bioconversion results. It is not clear at the conclusion of the project whether the chaperon system makes a significant difference or the improvement is just minor.

Work at IPB has focussed on biocatalytic steps towards phenylpropanoids, a major class of plant metabolites comprising valuable flavouring substances, antioxidants or phytoestrogens. Besides regiospecific introduction of hydroxyl groups, synthetic routes to these natural products rely on subsequent decoration by alkyl transfer (prenylation, O-methylation). IPB had 3'-specific catechol O-methyl transferases (COMTs), aromatic prenyl transferases (ubiA-type), and oxidases for a few selected substrates available. For a sustainable enzymatic production of these compounds which is competitive at industrial scale, solutions for the following tasks were required:

4.5 Development of suitable biocatalysts towards conversion of phenylpropanoids.

4.5.1 Oxidases: Expression studies were carrired out by ENTR using constructs carrying genes encoding three oxygenases from the oviedomycin gene cluster; OvmI, OvmII, and OvmIII. Genetic constructs, *E. coli* expression strains and expression protocols were transferred to Leibniz Institute of Plant Biochemistry (IPB) for testing against flavonoid substrates.

To generate a toolbox for conversion of phenylpropanoids and related compounds, a comprehensive screen of oxidising, specifically hydroxylating enzymes, was performed, including bacterial and human CYP450 enzymes and variants, flavin-dependent oxidases, laccases. Enzymes which were applicable for regiospecific hydroxylation of 25 compounds from a panel of 36 aromatics (e.g. phenols, flavonoids, cinnamic acids and anthraquinones) were identified. The most suitable enzymes, those with broad substrate specificity, were subjected to rational design, yielding variants of the flavin enzyme 4-hydroxyphenyl-acetate-3-hydroxylase with improved substrate scope and activity.

4.5.2 *O*-Methyltransferases (OMTs): Several plant OMTs appropriate for regiospecific methylations, i.e. for synthesis of (iso)vanilloid motifs from catechols or conversion of monophenols, were identified by screening. A spectrophotometric medium-throughput assay developed for subsequent optimization of catechol OMT activity, and a library of 48 variants of a plant OMTs was created. Selection of variants resulted in two enzymes with specificity for 3´-methylation, and fourfold increase in activity, along with a highly active variant with altered regiospecificity that allows production of isovanilloid compounds.

4.5.3 Aromatic prenyltransferases: The broad-spectrum prenyltransferase NphB proved to be an efficient tool for production of *O*- or *C*-geranylated phenols, flavonoids and coumarins.

Strategies to overcome substrate and cofactor limitation.

As application of prenyl- and methyltransferases in industrial processes is strictly limited by the availability of their co-substrates, synthetic routes to these costly compounds had to be developed. Natural (e.g. dimethylallyl and geranyl diphosphate) and artificial prenyl donors (e.g. homoallylic diphosphates with functionalization or heteroatom insertions) were generated in multi-gram scale and excellent purity from the corresponding alcohols, and tested for conversion by prenyltransferases (e.g. terpene cyclases). Furthermore, SAM synthase was engineered for production of the methylation cofactor SAM and artificial

analogues which might be useful in transfer of non-natural alkyl chains. This work forms the basis for a patent application by IPB (EP13005228.5).

4.6 Integration of biocatalytic steps.

For two-step biotransformation of phenylpropanoids, namely for production of mono-*O*-methylated catechols, whole-cell biocatalysts were developed. Live *E. coli* cells co-expressing the optimized oxidase and methyltransferase enzymes (see above) were used for the fermentative conversion of two monophenols - the stilbene resveratrol and the flavonoid naringenin – into the corresponding catechols and their downstream processing to (iso)vanilloid products. The tools and methodologies developed by IPB will be applied for the production of high-value synthetic phenylpropanoid compounds.

WORK PACKAGE *5*: *Fermentation Science*

The BIONEXGEN project was focused on the development, application and exploitation of a broad spectrum of tools and technologies. In this sense, the collaboration in WP5 of this EU project allowed a perfect mix between industry and academia, to introduce novel systems ready to be tested and used and to evaluate the feasibility of new industrial processes using biocatalysis.

Work Package 5 was perfectly described at the beginning of the project as an “enabling technology”. Together with Work Package 6, 7 and 8, one of the aims of our work, beside developing novel technology (in the case of WP5, involving process development and protein expression in efficient industrial hosts), was to support and enable BIONEXGEN member institutions to achieve their protein production goals in order to be able to test the enzymes of interest. As confirmation of such a role, numerous partners contributed to WP5 over the course of the project, in terms of proposed targets and collaborating activities; in turn, it was the responsibility of the led partner to extend and disseminate the available technology. In order to fulfil this role, researchers from USTUTT, GALAB and SUT were hosted by ACIB and Graz University of Technology facilities, for hands-on experience in protein expression and to support the design and construction of production strains for other BIONEXGEN partners. Besides hosting partner institution representatives, close interaction with all the members of the Work Package were frequent throughout the whole project.

5.1 Optimised expression of Cytochrome P450.

5.1.1 Multicomponent CYP: The first task of WP5 concerned the optimized expression of cytochrome P450s in microorganisms. In collaboration with USTUTT, BOKU Vienna (third party member of ACIB) tackled the task of optimizing the individual expression of CYP153 components (monooxygenase, ferredoxin, and ferredoxin reductase) in *E. coli* HMS174; identifying the bottlenecks in production of a three-component enzymatic system was of crucial importance to obtain a functional catalyst.

5.1.2 Catalytically self-sufficient fusion P450s: Additionally, USTUTT provided two fusion proteins of particular interest, where all of the catalytic components are produced as a single peptide. The first was the CYP153A from *Marinobacter aquaeolei* fused to CPR, the reductase domain of CYP102A1 (P450 BM3) from *Bacillus megaterium*. The second was CYP153A fused to PFOR, the reductase domain of CYP116B3 from *Rhodococcus rubber*. In cells expressing the fusion proteins, cellular metabolism was not disrupted during expression, in contrast to the expression of the multicomponent enzyme system where the increased metabolic burden of producing three enzymes was disruptive to the cell. Additionally, Hem1 from *Rhodobacter sphaeroides* (a biosynthetic enzyme for the production of haem which is required for the activity of P450 enzymes) was overexpressed under the control of an arabinose-inducible promoter without negative affect on cell growth in the bioreactor.

Co-expression of the P450 and Hem1, yielded 25mg_{CYP} $gCDW^{-1}$, corresponding to the highest CYP153 concentration so far obtained in fed batch. Fermentation scale-up of *E. coli* expressing CYP153A from *Marinobacter aquaelei* was performed for bioconversion reactions. Using dodecanoic acid for a linear-pulsed feeding of substrate, the maximum concentration of total terminally oxidized product was achieved after 27 h, with 1.39 g/L or 44.7 mg/gCDW. From this, 47% and 40% corresponded to 1,12-DCA (0.65 g/L) and 12-OHFAME (0.56 g/L), respectively (see WP2 for further details).

5.1.3 Fungal Cytochrome P450 expression: Together with bacterial P450s, fungal enzymatic systems were also explored, and in particular CYP52 from either *Candida tropicalis* or *Candida maltosa*. Such enzymatic systems, expressed as membrane proteins, consist of a monoxygenase and a reductase as electron shuttle, and are involved in oxygenation of linear alkanes. To express such eukaryotic enzymes, the methylotrophic yeast *Pichia pastoris* was used as a host. To produce a reliable expression system, both genes were placed on the same vector in a head-to-tail configuration and transformed into the yeast. In protein expression experiments with *P. pastoris*, since expression cassettes are integrated relatively randomly in the host genome, normally a large number of clones are screened. In the case of fungal P450, to be able to do so, a screening procedure based on whole-cell CO spectra was developed and published. Using such screening method, it was possible to identify high expression clones, which allowed exploration of substrate scope and bioconversions. However, the performance of the expressed enzymes was evaluated and found to be insufficient compared to other systems. Therefore other expression targets became a focus for activities at ACIB.

5.2 Galactose oxidase expression in yeast.

Support was provided for expression galactose oxidase variants, in collaboration with BASF and UNIMAN.

5.3 Bidirectional promoters for tuneable co-expression in *Pichia* pastoris.

In addition to such supporting activities, focus was applied to the development of novel expression strategies for the yeast *P. pastoris*. In many cases it is challenging to express enzymatic systems consisting of two (or more) polypeptides, and therefore efforts were

directed to develop a panel of bidirectional promoters, for the simultaneous control of transcription and expression of two gene products. The *Pichia pastoris* genome was mined to identify natural bidirectional promoters. This panel was subsequently expanded to include artificial fusions between known promoters, as well as novel promoters under development. The bidirectional promoter toolbox was initially tested using two different fluorescent proteins, to determine the absolute and relative expression levels of the two arms of the promoter combinations. Subsequently, several products of interest have been successfully expressed in such a system, including: the abovementioned fungal P450 system; CalB or variant galactose oxidase M3-5 co-expressed with PDI to facilitate their folding. The development of bidirectional promoters was the subject of an ACIB patent application in 2013.

5.4 Expression of enzymes for bioconversion of alcohols to amines.

In WP1 RUG established a multienzyme process for conversion of alcohols to amines. In WP5, overexpression of the enzymes required for amination reactions was performed. The alcohol amination scheme described in WP1 requires three enzymes: a transaminase, an alcohol dehydrogenase, and an alanine dehydrogenase for regeneration of the amine donor alanine from pyruvate. Expression levels of approximately 200 mg/L were obtained for several targets, codon-optimized for *E. coli* and expressed in either pET-28 or pBAD; such expression levels allowed successful implementation of the three-enzyme amination.

5.5 Knowledge transfer to BIONEXGEN partners.

Colleagues from partner institutions have been hosted on three occasions during the project. A brief *Pichia Hands-On* course was provided for GALAB.

During the last part of the project it was decided to support UCL and SUK to produce MAO-N in the methylotrophic yeast *P. pastoris*, to evaluate its potential. To do so, a three-way collaboration was initiated; UCL designed and ordered codon-optimized genes, and ACIB hosted representatives from SUK to perform expression studies in *P. pastoris*. Variant D5 was considered most interesting for application purposes, and therefore a mutation strategy was decided and successfully performed. The activity of MAO-N was tested against 3-Azabicyclo[3,3,0]octane HCl, and it was possible to identify clones showing up to 20 mU/mg of proteins. After optimization of protein production in a bioreactor (performed at SUK) it has been possible to optimize both protein expression and the whole-cell bioconversion process. Preliminary data indicate an activity of 0.8±0.09U/ml towards Azabicyclo[3,3,0]octane HCl.

At the beginning of the project, IMIC successfully obtained the production of α-L-Rhamnosidase in the license-bound *P. pastoris* strain GS115 (Life Technology, USA). As described in WP3, α-L-Rhamnosidase has the potential to be applied for value-added transformation of substrates for a wide market. At the end of the project, it was possible not only to transfer the expression cassette in a Free-To-Operate strain allowing wider exploitation of the process, but the yields of α-L-Rhamnosidase at least in small scale expression experiments were seemingly significantly higher than previously obtained.

As an enabling technology platform, we successfully supported several members of the BIONEXGEN consortium in establishing large(r) scale cultivations for effective proof-of-principle for bioconversion; moreover, we hosted and practically trained personnel from GALAB, SUK and IPB on protein expression in *P. pastoris*. Efficient expression clones for several BIONEXGEN enzymes including P450s, galactose oxidase, MAOs, α-L-Rhamnosidase, CalB, transaminases, ADH and more have been generated. An improved expression protocol for enzymes made by *Pichia pastoris* has been implemented. Such protocol is based on media optimization and on an accurate characterization of the carbon source specific strain requirement; we achieved a significant improvement, in terms of costs, performance and user convenience, of the BSM synthetic media for *P. pastoris*. Using our new procedure, the expression of two relevant enzymatic systems for Bionexgen, a functional fungal cytochrome P450 and a variant of galactose oxidase, was optimized.

A novel protein expression platform has been developed for facilitating expression of two polypeptides at the same time, and this activity brought scientific interest and international recognition, being reported at various international events. This work led to a patent application, and the system will be further optimized in upcoming projects, and commercialized by a planned start-up company of the inventors. This start-up company also secures the availability of the developed expression system beyond the duration of the BIONEXGEN project.

WORK PACKAGE 6: ***Biocatalyst Supports and Integration with Chemocatalysts***

The main goal in this work package was to develop practical methods for chemo-, and regio-selective aerobic oxidations of alcohols and polyols (including polysaccharides). Various enzymes and reactions have been investigated for this purpose: laccase, glucose oxidase, catalase, galactose oxidase, monoamine oxidase and rhamnosidase.

6.1 Oxidations using laccase.

Within WP6 CLEA focused on laccase and its combination with TEMPO as a chemocatalyst for alcohol, diol and polyol oxidations and developed practical methods for the selective aerobic oxidations of those. Starting with a thorough screen and selection of commercial laccases, an oxidation method was developed for starch and cellulose oxidation, as well as an immobilization procedure for production of laccase CLEA. These studies led to the delivery of the oxidase CLEAs and demonstration of their application, along with transfer of relevant know-how. A method for the aerobic oxidation of alcohols with oxidase CLEAs) has been delivered as a result of these studies. This work was performed in close collaboration with UNIOVI (WP4) for the application of laccase CLEA in the oxidation reactions of diols and we also calculated the costs and benefits of the enzyme catalyzed reaction versus the conventional chemical method(s).

The application of an oxidase (laccase) in combination with a chemocatalyst (TEMPO) has been investigated in detail and was reported in “Method for the aerobic oxidation of polyols (including polysaccharides)”.

Another potentially interesting application field for the laccase CLEA was the treatment of industrial waste water. Besides their primary application in alcohol and polyol oxidation reactions, laccases are known to have a significant potential for the several pollutants of environmental concern, including dyes (Figure 16), chlorophenols and endocrine disruptive chemicals. They have been applied for the decolourization and detoxification of effluents from textile, dyestuff, pulp and paper industries due to their highly unspecific oxidation ability. Laccase CLEA was successfully employed towards the conversion of five common industrial textile dyes. To this end, we also collaborated with LK and provided them a reaction procedure to test their candidate laccases in the decolourization project as well as an alternative *Trametes sp*. laccase to be used in their Saturn Blue L4G decolourization study (WP7).

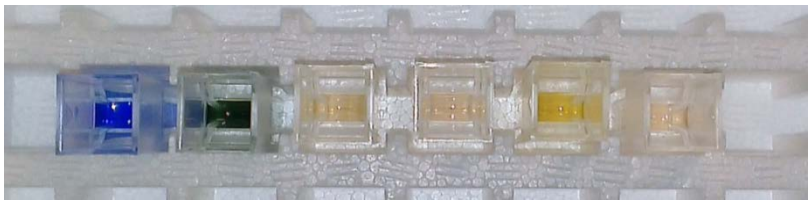


Figure 16: The overnight reaction of various laccases with the dye Saturn Blue L4G

6.2 Glucose oxidase.

CLEA examined glucose oxidase and its potential to oxidize glucose to glucaric acid, which is recognized by the US Department of Energy as one of the top "twelve building block chemicals" that can be subsequently converted to a number of high-value bio-based chemicals or materials. A glucose oxidase and catalase combi-CLEA was developed for this purpose, however we could not obtain glucaric acid as an end product from the oxidation trials we conducted. Nevertheless we investigated the applicability of glucose oxidase CLEA in large scale applications through our collaboration with DTU in WP7 and we assisted them for completion of practical reactor designs for use of CLEA oxidases.

6.3 Monoamine oxidase CLEAS.

MAO-N from UNIMAN was transferred to CLEA (via SUT) for the testing of its potential in CLEA. In WP 1, libraries of enzymes have been developed with the aim to be used as a technology platform in the synthesis of commercially relevant amines. UNIMAN and SUT collaborated on the immobilisation of *E. coli* cells expressing MAO-N variant D5 into a PVA matrix. CLEA worked with both partners to develop an alternative form of immobilization: immobilization of the cell free extract of MAO-N D5 as CLEA. At the end of this collaboration a straightforward protocol for the preparation of a MAO CLEA was developed. UCL studied the application of MAO CLEA for oxidation of 3-azabicyclo[3,3,0]octane. MAO was used as a whole cell biocatalyst for various applications within WP1, however

whole cell MAO has a short lifetime, both in storage and in use. By immobilizing the cell free extract of this enzyme as CLEA we significantly improved its stability, improving the lifetime of the biocatalyst from several days to several months (WP1 and WP7 reports for details).

6.4 Monoamine oxidase LentiKats® Biocatalyst.

The work of SUT within WP6 was closely linked to WP7 and WP8. The main goal of SUT in WP6 was to focus on immobilization of selected biocatalysts. In this field SUT intensively collaborated with SME partner LK, which is the industrial producer of polyvinyl alcohol lens-shaped particles for encapsulating biocatalysts, LentiKats® (Figure 17).

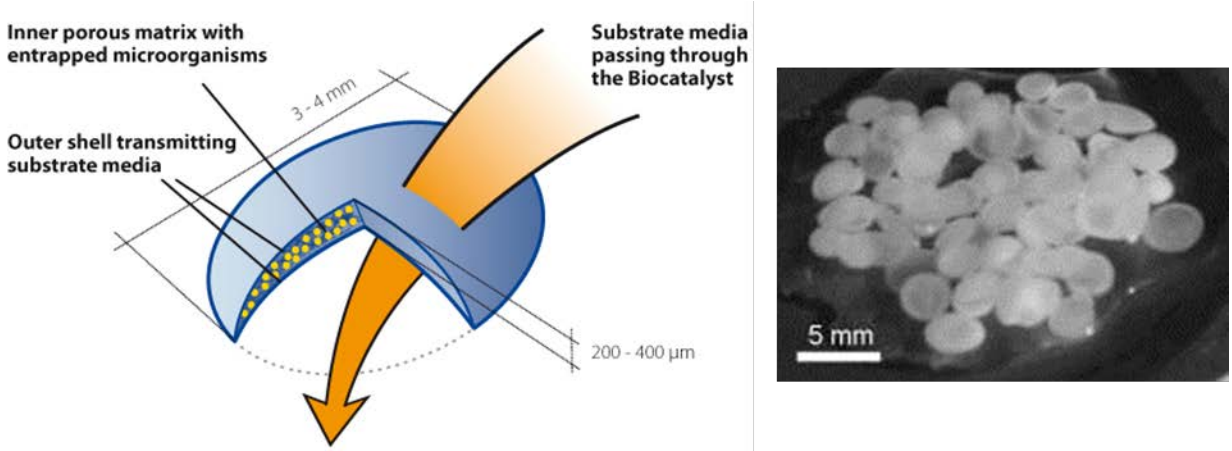


Figure 17: LentiKats® Biocatalyst

Lentikats Biotechnology offers several important benefits over other technologies, including relatively simple production procedure and easy separation of the Lentikats Biocatalyst particles (diameter 3 – 4 mm) from the reaction media. High enzyme activity yields after immobilization and also high biocatalytic activity are generally observed. The Lentikats Biocatalyst possesses excellent physical mechanical characteristics (elasticity, low abrasion) that provide for long-term stability and lifetime. Moreover, the polyvinyl alcohol (PVA) is biologically non-degradable and is nontoxic. It is an inexpensive matrix for immobilization of any biologically active materials with generally no side effects on biochemical processes. From the technical point of view, due to the high concentration of microorganisms or enzymes in the Lentikats Biocatalyst and the possibilities for reuse of the Lentikats Biocatalyst, a significantly shorter reaction retention time can be adopted, which minimizes size of bioreactors and leads to a concomitant reduction of infrastructure investment costs.

The main experimental model used in this WP was MAO-N provided by UNIMAN. SUT optimized and scaled-up the production of *E. coli* expressing monoamine oxidase MAO-N variant D5 to laboratory fermentors. Consequently, protocols for whole cell immobilization of biocatalyst to polyvinyl alcohol were developed and together with recommendations regarding the use of immobilized biocatalyst were made available for all partners. Additionally, a new approach for immobilizing high biomass concentrations (15 g_{DCW}/L_{PVA}) into LentiKats® was developed in order to provide higher activities per volume of biocatalyst.

For the study of immobilized MAO-N, large-scale fermentation of the whole cell biocatalyst was performed followed by production of 3kg LentiKats-MAO at the industrial pilot plant. The immobilized biocatalyst was made available to all partners.

Despite the initially high biomass concentration used for immobilization, five propagation steps of the Lentikats Biocatalyst in growth conditions prior to the use of the biocatalyst in biotransformation led to ~34% increase of the initial specific activity. Modifications of the commercially available fermentation equipment needed for the application of the immobilized biocatalyst in bioreactors were designed, enabling automated continuous working mode. The reaction loading with the immobilized biocatalyst was optimized to meet the technical requirements for potential scale up of the biotransformation. The whole cell immobilized biocatalyst was suitable for repeated use in biotransformations of the model secondary amine 3-azabicyclo[3,3,0]octane and retained its activity during long term storage. Based on the collaboration between SUT, UNIMAN and UCL, a student exchange project grant was successfully submitted. The research in this field will therefore continue, and will be expanded to study the MAO-N D9 and D11 variants, which possess alternative substrate scopes.

6.5 Laccase LentiKats® Biocatalyst for decolourization of textile dyes.

LK worked closely with SUT within WP6 and the research was focused on optimization of enzyme immobilization Lentikats Biocatalysts. The main goal was to immobilize redox enzymes and introduce a methodology for the determination and testing of immobilized oxidoreductase activity. The immobilized system was also tested in combination with a chemical catalyst. Laccases belong to an interesting group of multi-copper enzymes due to their ability to oxidize phenolic and non-phenolic compounds as well as some environmental pollutants. Because of their efficiency, laccases are a subject of interest for a variety of chemical industries, especially textile industry, where they are able to catalyze dye decolourization and result in colourless products. Dye decolourization of Saturn Blue L4G by immobilized commercial *Trametes versicolor* laccase, was compared with and without the chemical catalyst TEMPO, in both batch and continuous mode. In experiments with the laccase/TEMPO system in batch mode, the degree of decolourization was 10% lower, but the rate of decolourization threefold that of the reaction performed in continuous mode. The use of TEMPO was demonstrated to improve the process by enhancing the rate of dye decolourization five-fold compared to that of the laccase alone. The data obtained in experiments within WP6 were linked with work in WP7, and results were used for the final economic rating of laccase, reported in WP8.

Laccases immobilized into PVA matrix for removing/selective oxidation of dyes from aqueous solutions (wastewater) especially for use in textile industry were developed by LK. Despite a 40% decrease in activity over the course of one week, immobilized laccase (from *Trametes sp.*, provided by CLEA Technologies B.V.) was successful in decolorizing real wastewater from a textile plant.

Figure 18: Textile plant wastewater prior to decolourization.

WORK PACKAGE 7: *Bioprocess and Chemical Engineering*

The objective of WP7 was to take the next generation biocatalysts and reactions developed in WPs 1-4 and selected examples for evaluating possibilities and potential for scale-up. The reaction systems chosen as the focus of the WP were; transaminase (TAm), glucose oxidase (GOx), monoamine oxidase (MAO), rhamnosidase and laccase (LAC). Through excellent and intensive collaboration between different partners, several significant results were achieved within BIONEXGEN project. These results can be used for future application including industry. Early work by UCL focussed on TAm for the establishment of a methodology which was later used for other target systems. DTU similarly worked on MAO and GOx systems. LAC proved to be an industrially important example which was studied by LK, and CLEA laccases were also tested. MAO was chosen as the case study for establishing a methodology for process characterization, and subsequently studying process intensification.

7.1 Process technology handbook.

DTU's input in this work package started with analysing the myriad of options available for implementation of biocatalysis. An extensive literature review led to the creation of a process technology handbook which provides vital information for select process technologies in a single reference. The available information would help to provide the readers with a database of important tools available for use in biocatalysis and help them make educated decisions on how to effectively operate a process of interest. To complement the technology handbook, a process options matrix was developed. The primary objective of this matrix is to help match the reaction type and characteristics with the process technology tools that are available. The knowledge gained through compiling the handbook and process options matrix was then used to develop a framework for process characterization of a target oxidation reaction. The biocatalytic reaction of a secondary amine using a MAO was used as an exemplar for this work and the bottleneck for industrial implementation was established, in addition to suggestions of how the process can be operated. Notably, the framework can be extended for other oxidation reactions and also gives an insight to the factors affecting scale-up of bio-oxidation reactions.

7.2 Practical reactor design for application of CLEAs.

In addition to these studies, a close collaboration with CLEA Technologies was established. CLEAs of glucose oxidase were produced, and reaction characteristics were determined. Based on these results and previous knowledge of operating oxidation reactions, practical reactor designs for CLEA have been suggested. Scale-down reactors were designed and procured for use in these studies.

7.3 Data for evaluation of biocatalytic systems.

SUT performed modification of reactors to allow automation of repeated batch and continuous mode operations. This made the experimental system quite flexible for the rapid and effective generation data for processes carried out under various conditions. Two model biocatalytic systems were investigated by SUT; α-L-Rhamnosidase dase and MAO-N. The fungal exoglycosidase α-L-Rhamnosidase was developed by IMIC (WP3) and SUT in collaboration with LK for immobilization. The enzyme was successfully immobilized in LentiKats® and working parameters were defined for operational pH and temperature profiles, storage, and use over repeated reaction cycles. Critical to the viable industrial application of α-L-Rhamnosidase was scalable production of the biocatalyst using the expression strain developed by IMIC. The critical parameters for high-yield protein production were optimized, including the composition of the fermentation medium, induction phase, and induction substrate (methanol) feeding strategy. Together, the optimized production parameters achieved almost 36-fold activity compared to previously published results.

The second model system examined in this work was the variant monoamine oxidase, MAO-N D5, and experimental data were gathered with respect to: preparation of whole cell biocatalysts; crude enzyme extract preparation as cell-free biocatalyst; and immobilization of biocatalysts for improved process performance. These data formed inputs for economic assessments carried out in WP8 for the application of α-L-Rhamnosidase and MAO-N in biocatalytic processes.

7.4 Automated microscale methods for rapid evaluation of bioprocesses.

UCL developed automated microscale bioprocessing methods to enable rapid bioprocess design and development (Figure 19). These consisted of high-throughput microscale reaction systems, coupled to appropriate analytics. This allowed parallel screening of reaction process conditions in order to generate microscale biocatalyst performance data, including biocatalyst production and kinetic characterisation of enzymes.

In the work that followed, use of the microscale and kinetic modelling tools represented a 5-7-fold reduction in the number of experiments needed to obtain the full kinetic parameters or to optimize a fermentation and enzyme expression. It also represented up to 100-fold decrease in materials required compared to conventional laboratory techniques. In addition, using the microscale automated tools allowed for at least a 45-fold increase in experimental throughput. This represented considerable time and person-effort savings in comparison to manual and sequential laboratory scale approaches.

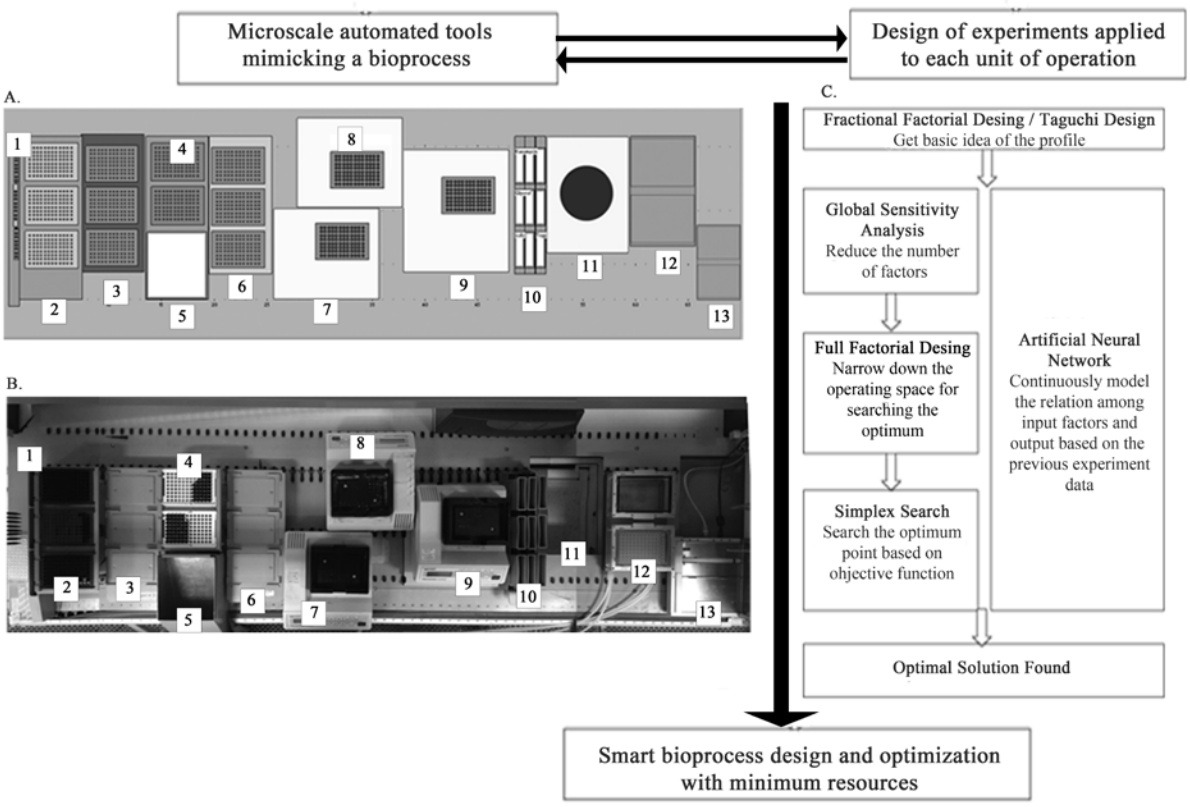


Figure 19: Schematic representation illustrating the linking of the microscale automated tools with the design of experiments algorithms in order to optimise and design a bioprocess with minimum resources: 1, Substrate reservoir; 2, Tips carrier; 3 & 6, Microplate carriers for bioconversions; 5, Waste reservoir; 7, 8, and 9, Fermentation microscale units; 10, Fermentation content reservoirs; 11, Microscale centrifuge; 12, Microscale filtration and low pressure device; 13, HPLC plate carrier.

7.4.1. Microscale evaluation of transaminase: The initial focus of UCL was to develop microscale methods to rapidly evaluate bioprocess options for increasing bioconversion yields in ω-transaminase-catalysed synthesis of chiral amines. This study allowed the implementation of improvements leding to a 3.3 fold increase in production yield (%mo/mol).

7.4.2. Microscale evaluation of MAO-N: Those microscale tools were then applied to optimize and scale-up MAO-N biocatalyst production, to obtain a six-fold increase in enzyme yield (%g/g) without loss of activity. After developing the expression and production of the enzymes, UCL developed microscale tools for high throughput screening and kinetic model generation of the enzyme. This work led to the discovery of interesting new substrates which upon MAO-N oxidation, would form new 5 and 6 membered carbon rings through bioconversions with great potential for commercialization.

UCL then adapted the microscale platform to study the solid-liquid suspension in shaken microwell bioreactors in order to quantify and determine its influence on immobilized MAO-

N enzyme kinetics. After determining the best conditions for solid liquid suspension using the microscale tools, UCL collaborated with CLEAs and Lentiktat's for kinetic characterization of both CLEA-MAO and Lentikats-MAO. Kinetic characterization was performed and the benefits of immobilization were evaluated in WP8.

7.5 Production of MAO-N as cell-free extracts foe encapsulation as LentiKats®.

Biomass production of *E.coli* expressing MAO-N was optimized by SUT in laboratory fermentors in batch mode. Crude enzyme extract production was optimized using a continuous cell disruptor. The prepared enzyme extract was immobilized in the form of LentiKats®. The immobilized MAO-N showed very good activity and improved stability, and the pH and temperature profiles were compared with those of the free enzyme extract in order to allow evaluation of these as alternative technologies.

7.6 Industrial application of enzymes immobilized as Lentikats Biocatalyst.

An important output of LentiKat´s a.s. (LK), as a combination of results WP6 – WP8, was the development and testing of industrially available enzymes - laccase from *Trametes sp.* (for decolourization of wastewaters with Saturn Blue L4G content) and recombinant α-L-Rhamnosidase from *Aspergilus terreus* expressed in *Pichia pastoris* (for production of isoquercitrin) during BIONEXGEN project. Two applications were developed by LK during the course of BIONEXGEN -decolourization of wastewaters by immobilized laccase and wine aroma release by immobilized rhamosidase into PVA matrix.

7.6.1. α-L-Rhamnosidase for isoquercitrin production: Rhamnosidases are biotechnologically important enzymes used for derhamnosylation of natural glycosides containing terminal α-L-rhamnose. Industrial applications include the debittering of grapefruit and other citrus juices by catalysing the hydrolysis of the bitter rhamnoside naringina, and aroma enhancement of wines and juices by the hydrolysis of terpenyl glycosides.

Rutin and isoquercitrin possess relatively low aqueous solubility (for rutin 1.25 g/L), and during the covnersion reaction an equilibrium is established which allows simultaneous dissolution of substrate and crystallization of product. The advantage, therefore, of using free enzyme for bioconversion is the possibility of using high substrate concentrations in order to significantly shift the equilibrium in favour of product formation. Additionally, the by-product rhamnose is soluble to 300 g/l at 25 °C, and therefore remains in solution in the bioconversion broth, and may be simply washed out of isoquercitrin crystals after reaction.

Unfortunately, due to the unfavourable rheological properties of mixtures containing high concentrations of rutin and isoquercitrin, this strategy to drive the equilibrium in favour of product formation cannot be performed with Lentikats Biocatalyst. Therefore LK's studies were mainly focused on enzyme stability in the immobilized form, and the ability of substrates and products to diffuse through the PVA matrix at low substrate concentrations (2 mM), and these data will also inform other applications.

Rhamnosidases immobilized into a PVA matrix were developed in the BIONEXGEN project in cooperation with SUT for application in the enhancement of the most important characteristic of a quality wine; its aromatic fragrance. Recombinant α-L-rhamnosidase immobilized as Lentikats Biocatalyst provides a stable and cheap enzymatic technology for improving wine aroma.

7.6.2. Laccase Lentikats for wastewater treatment: Application of Lentikats Biocatalyst with immobilized laccase was tested for removal of various dyes from model systems, and also for decolourization of Saturn Blue L4G dye in authentic industrial wastewater. Based on chemical engineering calculations, a system was designed for implementation by an industrial end-user for the decolourization of textile manufacturing wastewater, using 3 batch reactors working as SBR (Sequence Batch Reactors). Process systems for wastewater pumping, reactor mixing, Lentikats Biocatalyst separation from liquid media and CIP (Cleaning In Place) were designed. The complete technology solution was presented to potential end users, including process economy assessments performed in WP8 with comparison to systems using free enzymes or traditional chemical methodologies. It is hoped that this solution will be widely adopted by industry for the remediation of waste streams.

WORK PACKAGE *8*: *Economic, Environmental and Life Cycle Analysis*

The objective of WP8 was to evaluate the processes developed in WP1-4 and developed further in WP7 and 8 in terms of their economic competitiveness and environmental impact.

8.1 Process modelling bioconversion of alcohols to amines.

Process modelling and evaluation were carried out by DTU for a multi-enzymatic system for the synthesis of chiral amines identified in WP1 and thus, the results obtain in WP8 were for the system resulting from the collaborations between RUG and BASF. The result of this collaboration was a set of guidelines for process development, where process challenges (such as unfavourable thermodynamic equilibrium) can be overcome and thus, the process can be successfully operated. To obtain the operating space, where reaction yield and final product concentration can be achieved, it is necessary to collect thermodynamic data and information about the upper boundary for the maximum concentration of co-substrate that can be used, as well as the lower concentration of product and/or co-product achievable in the aqueous solution by implementing a ISPR and/or IScPR technology, respectively. In this work package the main bottlenecks for the three-enzyme process where identified, where the unfavourable thermodynamic equilibrium might hinder the successful scale-up due to the low

yield at equilibrium. Hence, using window of operation, a feasibility study to identify the potential process technologies that must be put in place to shift the thermodynamic equilibrium was done.

8.2 Tools for design and implementation of strategies towards biocatalytic processes.

Despite the potential economic and environmental benefits of adopting a biocatalytic route, such a conclusion is not justified without the use of adequate tools to evaluate the performance of a process, in particular during process development. A thorough analysis at the early development stage (as is the case for the biocatalytic reactions in BIONEXGEN) is further complicated by a lack of data or data from non-optimised systems. Hence, a range of tools were proposed which can guide process development, research tasks and support decision-making throughout the different development stages of a process. Three sets of metrics were identified, each for use at different stages of process development and design (route selection, early development and late development stage) and each with different objectives: to eliminate unfavourable routes, set target for biocatalyst and process development and compare and evaluate different developed technologies.

8.3 Evaluation of enzymatic process for isoquercitrin production.

Within this WP there was intensive collaboration between SUT and LK. This cooperation resulted in evaluation of academic research results to the real, industrial evaluation of developed technologies. The first period was mainly focused on creation of the databases required for evaluation of three relatively mature enzyme systems: laccase, monoamine oxidase and α-L-rhamnosidase. The databases summarized all of the available data concerning substrates and products, and also the enzymes themselves.

Next SUT generated process data for selected experimental models: monoamine oxidase and α-L-rhamnosidase. Due to the industrial interest in α-L-rhamnosidase and its application for the pharmaceutical and beverage industries, the main focus of SUT was on this enzyme. Date was generated for three technologies developed separately: 1) production of biocatalysts: α-L-rhamnosidase fed batch production by *Pichia pastoris*; 2) immobilization of the enzyme; 3) Isoquercitrin production. Research in each separate part was focussed on optimization of each separate step and its process development. The data gathered served as crucial inputs for economic evaluation and feasibility estimations of the developed processes. These calculations were made in cooperation with LK, which estimated the economy of all processes according to actual costs in Czech Republic. Economic evaluations of these results were summarized with comparison to conventional chemical routes.

Within WP8, LK were focused on preparation of databases with information on substrates, products and by-products of reactions used in BIONEXGEN project. One of the main tasks was the preparation of economic data for Lentikats Biocatalyst with immobilized recombinant α-L-rhamnosidase from *Aspergillus terreus* (expressed in *P. pastoris*; WP5) for isoquercitrin production.

8.4 Economic assessment of wastewater treatment processes using immobilized lacasse.

An important output from WP8 was the economic rating of the process using Lentikats Biocatalyst with immobilized laccase from *Trametes sp.* for real wastewater decolourization (WP6), with comparisons to conventional chemical routes (Fenton's reaction) or the free enzyme (from *Trametes sp.*). Laccases belong to the group of multi copper enzymes, with industrial relevance due to their ability to oxidize phenolic and non-phenolic compounds as well as some environmental pollutants. Because of their efficiency, laccases are of interest for a variety of chemical industries, particularly the textile industry, where they are applied to dye decolourization. Laccases immobilized into polyvinyl alcohol (PVA) matrix for removing/selective oxidation of dyes from aqueous solution (wastewater) especially for use in textile industry were developed by LK.

Laccase from *Trametes* sp. (CLEA WP4) immobilized as Lentikats Biocatalyst was applied to wastewater decolourization (WP6). Data showed that the activity of the laccase decreased by 40% during one week of continuous operation, and thus, this was defined as the operation lifespan of the catalyst. On this basis, comparison was made of the economic parameters for dye decolourization by Lentikats Biocatalyst versus the conventional chemical route (Fenton's reaction) or *Trametes* sp. free enzyme preparations (Table 1).

The work on wastewater treatment in WP6-8, led to the final methodology proposed by LK: *Decolourization of industrial wastewater by laccase immobilized into PVA matrix.*

Table 1: Economic parameters for dye decolourization by Lentikats Biocatalyst with immobilized laccase (from Trametes sp.) in contrast to conventional chemical route (Fenton's reaction) or free enzyme (from Trametes sp.)

Annual operational costs estimation	**EUR/year**
Chemicals consumption – Fenton's reaction (for treatment of 360 000 m^3 wastewater per year)	506 600
Free laccase, other chemicals	13 757 625
Lentikats Biocatalyst with immobilized laccase from *Trametes versicolor*	488 000

8.5 Application of microscale experimental data for improved MAO-N reaction screening.

UCL used microscale experimental methods developed in WP7 to obtain kinetic data of the model reaction as well as a range of other substrates. This data would be then used by the other WP8 members to perform the environmental and economic analysis of the selected process. UCL applied the microscale tools to develop kinetic models for oxidation of the model substrate of MAO-N. Excellent agreement was found between the predicted and experimental data. High throughput screening was then performed over a broad range of compounds, leading to identification of the novel substrates described in WP7.

The kinetic models of those new substrates were developed, and laboratory scale-up was performed with excellent agreement between the experimental and predicted data. UCL then adapted the microscale platform to study solid-liquid suspensions in shaken microwell bioreactors, in order to quantify the effects and determine influence on immobilized enzyme kinetics. After determining the best conditions for solid-liquid suspension using the microscale tools, UCL collaborated with CLEA and LK for kinetic studies. The complete kinetic model of the MAO-N Lentikats Biocatalyst was obtained and validated. Integrating the fermentation, downstream, screening and kinetic steps in a single automated platform enabled the discovery of new products synthesised by MAO-N-D5, while simultaneously revealing the bottlenecks and engineering principles required in order to make the process economically feasible.

PUBLISHABLE SUMMARY Please provide a description of the main S & T results/foregrounds. The length of this part cannot exceed 25 pages

Key results:

WP1
1. A toolkit for chiral amine synthesis.
1.1 Hydroxynicotine oxidase.
1.2 Imine Reductases.
1.3 Dynamic kinetic resolution using IRED and MOA-N/HDNO.
1.4 Kinetic resolution and asymmetric amination using Phenylalanine aminomutase.
1.5 Biocatlytic evaluation of alcohol to amine conversion using a multienzyme approach.
1.6 Novel bio-amination cascade.

WP2
2.1 Cytochrome P450 monooxygenase-catalyzed terminal hydroxylation of fatty acids.
2.2 Biocatalytic production of medium-chain dicarboxylic acids.
2.3 Biocatalytic production of medium chain fatty acids and corresponding ω-hydroxylated derivatives.
2.4 Enzymatic routes toward bio-based functional polymers and cross-linked materials.

WP3
3. Developing enzymes for oligosaccharide synthesis.
3.1 Novel enzymes for synthesis of oligosaccharides.
3.2 Synthesis of oligosaccharides using starch as a renewable starting material.
3.3 Modification of natural polysaccharides to improve properties.
3.4 Transglycosylases.
3.5 Gylcosyltransferases
3.6 α-L-Rhamnosidase for selective trimming of rutin.

WP4
4.1 Glucose oxidase/catalase combi-CLEA
4.2 Laccase CLEA
4.3 Oxidation of diols and amino alcohols.
4.4 Tryptophan halogenases.
4.5 Development of suitable biocatalysts towards conversion of phenylpropanoids:
4.5.1 Oxidases.
4.5.2 *O*-Methyltransferases (OMTs).
4.5.3 Aromatic prenyltransferases.
4.6 Strategies to overcome substrate and cofactor limitation.
4.7 Integration of biocatalytic steps.

WP5
5.1 Optimised expression of Cytochrome P450.
5.1.1 Multicomponent CYP.
5.1.2 Catalytically self-sufficient fusion P450s.
5.1.3 Fungal Cytochrome P450 expression.
5.2 Galactose oxidase expression in yeast.
5.3 Bidirectional promoters for tuneable co-expression in *Pichia* pastoris.
5.4 Expression of enzymes for bioconversion of alcohols to amines.
5.5 Knowledge transfer to BIONEXGEN partners.

PUBLISHABLE SUMMARY Please provide a description of the main S & T results/foregrounds. The length of this part cannot exceed 25 pages

WP6

6.1 Oxidations using laccase.
6.2 Glucose oxidase.
6.3 Monoamine oxidase CLEAS.
6.4 Monoamine oxidase LentiKats® Biocatalyst.
6.5 Laccase LentiKats® Biocatalyst for decolourization of textile dyes.

WP7

7.1 Process technology handbook.
7.2 Practical reactor design for application of CLEAs.
7.3 Data for evaluation of biocatalytic systems.
7.4 Automated microscale methods for rapid evaluation of bioprocesses.
7.4.1. Microscale evaluation of transaminase.
7.4.2. Microscale evaluation of MAO-N.
7.5 Production of MAO-N as cell-free extracts foe encapsulation as LentiKats®.
7.6 Industrial application of enzymes immobilized as Lentikats Biocatalyst.
7.6.1. α-L-rhamnosidase for isoquercitrin production.
7.6.2. Laccase Lentikats for wastewater treatment.

WP8

8.1 Process modelling bioconversion of alcohols to amines.
8.2 Tools for design and implementation of strategies towards biocatalytic processes.
8.3 Evaluation of enzymatic process for isoquercitrin production.
8.4 Economic assessment of wastewater treatment processes using immobilized lacasse.
8.5 Application of microscale experimental data for improved MAO-N reaction screening.

PUBLISHABLE SUMMARY Please provide a description of the potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and the exploitation of results. The length of this part cannot exceed 10 pages.

The Potential Impact of BIONEXGEN

The development and uptake of biocatalytic processes by the chemical manufacturing industry is vital for expanding the European knowledge-based bio-economy (KBBE). The development of next generation biocatalysts will allow the rapid adoption of new technologies which deliver improved performance over conventional processes, with concomitant benefits to efficiency and economy. The use of enzymatic manufacturing methodologies has the potential to 'green' industrial chemical production by displacing conventional chemistries which require toxic and hazardous reagents, such as metal catalysts and strong acids, and energy-intensive processes operated at high temperatures and pressures. BIONEXGEN will help to develop the research and design skills base of the participating scientists, increase the expertise of the partner organisations, grow an active network of collaborators, and deliver new technologies for industrial application.

Technology Overview and expected impact

BIONEXGEN has delivered new technologies for the synthesis of monomers from renewable sources, and their enzymatic polymerisation for production of materials with improved characteristics:

1. Enatiocomplementary enzymatic toolkit for synthesis and modification of chiral amines
2. Biocatalytic cascades for synthesis and dynamic kinetic resolution of amines
3. Synthesis of and development of fatty acid monomer to expand the operative range of green polymers
4. Functionalization of the polymeric main chain via functional group metathesis during polymerization
5. Fatty acid-based monomers incorporated into the portfolio of products
6. Novel fermentation technologies as well as polymerisation chemistry utilising abundant, inexpensive, and renewable feedstocks
7. Tools for synthesis of glycans, e.g. human milk oligosaccharides, and modification of polysaccharides
8. Enzymatic manufacturing process for production of isoquercitrin
9. Enzymatic transglycosylation processes
10. New immobilization technologies and know-how for implementation in industrial processes
11. Commercialised Laccase CLEA
12. Biooxidation processes for improved safety, environmental performance, and novel product manufacture
13. Application of laccases for production of high-value lactams and lactones
14. Biohalogenations for production of high-value building blocks
15. Toolkit for preparation of modified phenylpropanoids for applications in flavours and fragrances and regeneration of cofactors

16. Improved fermentation technology for production of proteins from *E. coli* and *P. pastoris* hosts
17. Bidirectional promoters for tuneable co-expression of multiple proteins in *P. pastoris*
18. Immobilized α-L-Rhamnosidase for enhancing food and beverage flavours and aromas
19. Laccase Lentikats Biotechnology for wastewater treatment
20. Integrated bio- and chemo-catalytic processes
21. Process engineering methodologies for application of biocatalytic methodologies
22. Microscale technologies for rapid determination of bottlenecks, and process optimization
23. Process engineering handbook
24. Performance metrics for environmental analysis and economic evaluation of bioprocesses
25. Reaction databases for comparison of bio- and chemo- manufacturing processes

Amines are amongst the most important building blocks for agrochemicals, polymers, fine-chemicals and pharmaceuticals. Conventional technologies for the synthetic production of amines are often wasteful and require harsh reaction conditions. Chemical routes to chiral amines can be particularly challenging, and many molecules remain completely inaccessible. Traditional manufacturing methodology for the production of chiral amines often require deracemisation of products, therefore limiting yields to a maximum 50%, since the unwanted enantiomer is not required. Dynamic kinetic resolution of racemic mixtures and other strategies can increase yields to >50%. Crucially, the use of biocatalytic routes offers greater potential for the direct asymmetric synthesis of chiral amines. The work in BIONEXGEN provides direct synthetic routes to enantiopure amines from readily available, cheap feedstocks, such as alcohols. The biocatalytic cascade systems developed in BIONEXGEN allow the selective functionalization of products using simplified processes with minimal requirements for cofactor supplementation. These have the potential to complement traditional chemical routes to complex products, and often to displace conventional technologies entirely to provide cleaner, safer processes. Patent applications have been submitted for two alternative biocatalytic amine synthetic cascades, and it is anticipated that these new routes can be developed for application with significant advantages over conventional syntheses.

Demand for bio-based materials is ever growing, offering new resource-based economic development opportunities for manufacturing sectors. However, many of the technological and economic benefits are not inherent to the material, but require that bio-based products meet minimum standards for safe production, use, and end-of-life recycling and disposal. The transition from a petroleum-based to bio-based economy presents a unique opportunity to re-evaluate product standards so that sustainability and minimised environmental impacts, human health, safety, and improved performance are inherent features of new materials. The new methodologies developed in BIONEXGEN represent important steps towards delivering those benefits. However, the benefits of bio-based polymers must be maximized without risking their commercial viability, and this is likely to require an ongoing combination of

policy incentives and regulation, private-public engagement and support, and market development which supports economic, environmental and social objectives.

The potential of modified oligosaccharides is well recognised for applications in bio-based and bio-degradable polymers, polymerizable building blocks, surfactants, and rheology modifiers, amongst many others. The development of tools for selective synthesis of glycans will open new horizons for their application in the fields of human health, food, and materials. BASF plans to submit a patent for the applications developed within BIONEXGEN. The improvement of infant formula nutrition remains a challenge, but the development of tools which can be applied for production of human milk oligosaccharides is an important output of BIONEXGEN. An efficient and simple biocatalytic route to the production of isoquercitrin was established using the enzyme α-L-rhamnosidase. This compound attracts significant interest as a nutraceutical and potential pharmaceutical, with activity described towards a host of conditions including, cardiovascular disease, asthma, stroke, capillary fragility, arteriosclerosis, hypertension, type II diabetes, and Alzheimer's disease. This compound is now being used for research purposes, but further application and evaluation (through animal studies) has been hindered by its high market price. The methodology developed within BIONEXGEN provides high-purity isoquercitrin at an affordable cost, and in sufficient quantities to satisfy high demand for this value-added product. Mother's milk is one of the most complex foods, with all of the key ingredients for the healthy nutrition of babies. Growth, intestinal and brain development, and strengthened immunity are all benefited by feeding with human milk. Targeting these structures requires highly selective methods to convert cheap renewables to functional materials for industries such as cosmetics and pharmaceuticals. Tools like enzymatic transglycosylation are highly attractive for their inherent functional group tolerance and selectivity.

Chemical oxidations are often hazardous and polluting, and limited in scope. In BIONEXGEN, new biocatalytic routes were opened for syntheses of otherwise challenging or inaccessible products, targeted towards building blocks for the pharmaceutical industry as novel APIs, and flavonoids with new patterns of oxidation not found in natural resources. Within the BIONEXGEN project, immobilized biocatalysts (laccase CLEAs) were produced to be employed in newly developed practical methods for oxidation reactions with the focus on alcohol and polyalcohol oxidations. A number of different enzymes were screened for this purpose and selected candidate enzymes which were then further developed into immobilized biocatalysts. While developing methods, industrial application criteria were taken into account, such as price, safety, selectivity, suitable reactor types and re-usability of the catalyst. Environmental and economic assessment of the chemical and enzymatic oxidation approaches were performed, demonstrating that enzymatic catalysis offers significant advantages such as higher specificity, ambient reaction conditions, and generation of less waste when compared to conventional methods in oxidation reactions. In addition to these benefits, catalyst recyclability has been demonstrated when the enzymes are used in immobilized form. The outcome of this assessment also showed that, besides its environmental gains, the enzymatic approach can be cost-efficient and can deliver a financially competitive alternative to the chemical methods. These novel methodologies have

been presented to different industries, inside and outside BIONEXGEN, and evaluated for their future applications.

Halogenated compounds are important classes of bioactive compounds, for instance, chlorinated drug molecules may be resistant to metabolism by the human liver, thus maintaining the active compound in the bloodstream, and avoiding generation of toxic metabolites. Therefore, medicinal chemists and pharmacologists often design target molecules incorporating chlorine. However, these designs are often difficult to implement, since chemical halogenation is limited, and obliges Chemists to start with more basic building blocks, therefore requiring more chemical steps (and more contamination) to reach the target molecule. The halogenases developed in BIONEXGEN represent a step towards overcoming these challenges, and although the technology has not yet been realised to allow industrial production, will allow the manufacture of halogenated compounds at preparative scale for initial research purposes.

BIONEXGEN has produced toolboxes for preparation of modified phenylpropanoids: a set of enzymes for (I) hydroxylation of a broad range of aromatic compounds, and (II) for the regioselective methylation of phenols, including the highly selective generation of (iso)vanilloid motifs from catechols. Advantageously, these enzymes proved to be applicable not only as single enzyme systems but also in cascade reactions and in fermentation (whole-cell biocatalysis). Furthermore, improved enzymes for (re)generation of the cofactor SAM were developed, and a patent application has been submitted to protect this invention. The economic significance of these biocatalysts which facilitate a sustainable production of high-value phenylpropanoids is reflected by the strong interest of industrial partners in their application.

Alongside the development of next generation biocatalysts, BIONEXGEN also delivered the vital enabling technologies to allow rapid development of enzymes and biocatalysts, their integration into processes, and economic and environmental evaluation versus conventional and competing technologies.

Reliable and substantial enzyme production are key to opening the way for further process development, and must be considered from the earliest stages of the evolution of those processes. Without those capacities, development of processes can be difficult, and their implementation at scale impossible. New technologies for improved protein production were developed and implemented for the biocatalysts used across BIONEXGEN, including optimised fermentation methodologies, and improved strains and media for enzyme expression in both *Escherichia coli* and *Pichia pastoris*. A novel platform was developed for the production of complex enzymatic systems in the yeast *Pichia pastoris*. The requirement for expression of multienzyme systems, composed of two (or more) enzymes is becoming increasingly commonplace, as novel enzymatic cascades are implemented. Hence, a suite of promoters capable of expressing two enzymes at the same time in the yeast (or, even more interestingly, in a pre-determined order of expression) were discovered, cloned and tested, and this invention is the subject of a patent application. This innovation is expected to

provide an enormous impact in the fields of synthetic biology and biopharmaceutical protein production, as it facilitates the coupled production of polypeptides, for example for production of functional Cytochrome P450s-reductase systems.

Enzyme immobilization is a key enabling technology for the development of industrial biotransformations, facilitating the recycling and reuse of the biocatalyst. Cross-linked Enzyme Aggregates (CLEAs) consist of covalently cross-linked precipitated enzymes. This efficient and economically attractive method yields immobilized biocatalysts that do not include support material and therefore have a very high activity per unit volume. This methodology allows the biocatalyst to be tailored to suit the application. Physical characteristics such as particle size and hydrophobicity can be tuned for applications in organic media, and moreover, smart functions like magnetisability can be introduced for special applications.

Practical methodologies and manufacturing processes have been developed for the production of a number of enzymes developed in BIONEXGEN as immobilized enzymes, including laccase (as CLEAs and Lentikats Biocatalysts, but also LentiCLEAs), MAO-N (as CLEAs and Lentikats Biocataltalyst), and α-L-Rhamnosidase (as Lenitkats Biocatalyst). Of these, laccase CLEAs have been made commercially available, and immobilized α-L-Rhamnosidase was evaluated by a Czech wine company for aroma enhancement, and further offered to beverage and pharmaceutical companies. A practical process was designed for the application of laccase Lentikats Biocatalyst in the treatment of wastewater contaminated with dyestuffs. This market-ready technology presented, including practical reactor design and economic evaluation, as a solution for a customer in the textile manufacturing industry.

Two of the biggest work packages (WP7 & 8) in this project were to strengthen the basic understanding of process engineering and develop a knowledge-base that would underpin research strategies, process development, and the implementation of practical, cost-effective, efficient methodologies. A process technology handbook was compiled, integrating state-of-art reports to provide the readers with a database of important tools available for use in biocatalysis. To complement the technology handbook, a process options matrix was developed to help match the reaction type and characteristics with the process technology tools available. The knowledge gained through compiling the handbook and process options matrix was then used to develop a framework for process characterization of a target oxidation reaction. Automated microscale bioprocessing methodologies were developed in order to rapidly develop processes. These methods facilitate data collection from parallel reactions in order to test biocatalyst performance under varying reaction conditions, identify bottlenecks and generate data for process evaluation. These methodologies reduce screening efforts, thereby reducing person effort and resources required. These methods were applied for the development of biocatalysts in BIONEXGEN, but have broad applicability, with the potential reduce costs and time for the development of novel bioprocesses. Economic and environmental metrics were developed in order to provide a practical basis for comparison of competing technologies. These tools would provide a basis for the design of research and

development strategies, from the earliest stages of projects through to the implementation of processes.

Overall, BIONEXGEN has delivered new knowledge and technologies with the potential to transform manufacturing processes and shape future industrial biotechnology research in Europe. The know-how and applications resulting from this flagship collaboration may be integrated into manufacturing workflows of the current portfolio of chemical products, but could also supersede many of the conventional inefficient, polluting, and hazardous methodologies. Excitingly, in many cases these new technologies also allow access to completely novel products that were not previously realisable. This has the potential to not only change routes for chemical manufacture, but also to introduce new functionality and improved product performance.

Impact on Research Infrastructure

One of the main objectives of the BIONEXGEN project was to gather manufacturers and research organisations from across Europe, bringing together the necessary interdisciplinary skills in order to form a strong transnational collaboration to generate new knowledge and new technologies that will be implemented in existing but also, importantly, new products. BIONEXGEN will contribute to sustain the European research and boost collaboration with universities, thus allowing European research organizations and European industries to achieve and provide leadership. The success of the project can be judged by the impressive body of high-impact literature generated, but also by a lasting legacy of enhanced cross-sector collaboration. By promoting close collaboration between academe and industry, BIONEXGEN placed the requirements of industry at the heart of the project, leading to the development of advanced research skills to tackle real-world problems for commercializing biocatalytic reactions.

The success of BIONEXGEN can also be illustrated by some examples of the enhanced research infrastructure which will outlive the lifespan of the project. The University of Manchester has acted as coordinator for a number of EU projects, and by continuing to act in this role, has developed expertise for the management of large, complex trans-European research projects. The effective management of projects is key to the successful delivery of research outputs, and the benefits of this capability will continue in ongoing and future EU research projects. Continuing research collaborations have been strengthened and established, for instance, a number of BIONEXGEN partners (UNIMAN, USTUTT, DTU, CLEA, BASF) will form part of the consortium of the new FP7 project BIOOX (613849). Such examples of sustained cooperation can be found across the consortium.

BIONEXGEN and the new knowledge that it generated will attract young scientists to join the biotechnology industry. The project will be a source of highly trained personnel who will remain in Europe thanks to the increased demand for qualified people in industry and academia. The joint meetings and exchanges throughout the project of young researchers for training have helped them expand their skills and knowledge of industrial biocatalysis along

with the critical enabling technologies, and thereby contributed to an increasing the knowledge base in Europe. ACIB have provided training and knowledge transfer in practical methods for protein production, one of the key enabling technologies for biocatalyst development and application, helping to address the apparent skills shortage within this specialised area. Young researchers from RUG, DTU, and UNIMAN have interacted closely with BASF in order to deliver processes for the production of amines which are currently the subject of patent applications. These young researchers will become future research leaders, and by increasing their understanding of the needs of industry and future challenges for biocatalytic manufacturing, BIONEXGEN has help to shape the future direction of industrial biotechnology in Europe.

UCL have collaborated throughout the consortium, but particularly with DTU, SUT, and LK to develop robust tools for the rapid analysis and evaluation of bioprocesses which will help to reduce the effort and resources required for future research in this area. The development of broadly applicable metrics and methodologies with WP7 and 8 will also allow the implementation of more effective research strategies and earlier evaluation of developing technologies in order to better reflect the requirements of the industrial applications.

Participation in a flagship project such as BIONEXGEN not only increases networking opportunities and resources available for partners via collaboration within the consortium; UNIOVI, for instance, has interacted with individuals from different countries around Europe in industry or academia not only inside WP4 (EntreChem, CLEA) but also outside (University of Barcelona). The high-impact results from UNIOVI have been presented in international conferences (EMBO Groningen 2013; Biotrans Manchester 2013; Brussels Event 2013), further increasing the international recognition and reputation of their research.

Efficient technology transfer from academic laboratories to European industrials is also crucial to ensure the competitive development of the chemical industry in the European countries. For example, the results made available from USTUTT, KTH and others in BIONEXGEN will push the state of the art of biopolymer synthesis and it is expected that this will have continuity and synergy within Horizon 2020, the successor of the 7th Framework Programme for Research and Development, alongside the implementation of findings by European companies. IPB have delivered important new technologies which will benefit industry in the form of commercialized processes for flavonoid production, whilst also increasing the fundamental knowledge within that field. Industrial partners profit from the fruitful discussions with scientific and other industrial partners. This allows the contributing institutions to establish techniques and raise competence in the field of biocatalysis. Biocatalysis is one of the most promising techniques in food and nutraceuticals. Synthetic oligosacharides developed within WP3 (UNIMAN, GALAB, IMIC, BASF) are just one example where biocatalysis is only viable route to the products.

BIONEXGEN has also supported emerging researcher leaders to gain experience in EU projects. Participation in the project has allowed SUT to apply for a structural funds project in Slovakia (wherein participation in an FP7 project was an essential criterion). The new

collaborations established between SUT and leading research institutions have enhanced its reputation through its participation in BIONEXGEN, which helped it to secure national (APVV grant agency) and also international (COST Action "Systems Biocatalysis", new FP7 GRAIL) research funding.

Socio-economic Impact

The new technologies and methodologies developed within BIONEXGEN will provide a legacy of benefits which will help to maintain the competiveness of the European industrial biotechnology community.

An enhanced skill base will provide benefits to the research infrastructure, but also provided vital expertise for the implementation of biocatalysis by industry. Enzymatic routes have the potential to improve the economic performance of many industrial manufacturing processes, by increasing efficiency (for example by providing high-yield asymmetric syntheses of amines), reducing the requirement for expensive metal catalysts (particularly as demand grows for rare metals in other products and processes), and reducing waste generation and the associated safe disposal costs. The decreased energy demands of many biocatalytic processes, using lower temperatures and pressures, will also similarly lower the costs of manufacturing. Novel products, made accessible by these new processes have the potential to add value to otherwise low-value feedstocks, and can reduce the requirement for increasingly expensive fossil resources. Since sustainable resources can alleviate demand on petrochemicals and gas, the use and uptake of such renewables will also increase resource and energy security in Europe by reducing demand on sources outside the EU. Decreased costs will improve the economic competitiveness of European chemical manufacturing at a time when there is increased competition from economic areas outside the EU with lower inherent infrastructure and personnel costs. This will help to secure current and future jobs within the EU, and requirement for state-of-art technology and know-how will ensure that the IP is further protected.

The European IB community has a reputation as global leaders in innovation and expertise. Projects like BIONEXGEN, which deliver skilled and knowledgeable researchers, along with new applications, methodologies, and products, help to enhance and sustain that position. The status of the EU as a prominent KBBE attracts inward investment in European technologies and expertise for their implementation across the world.

A number of technologies developed in BIONEXGEN are moving quickly towards commercial realisation. The variants of the SAM synthase developed by IPB have formed the basis for a patent application (EP 13 005 228.5), and will be commercially realized via industrial partnerships. IPB plans two more patents based on work performed in BIONEXGEN. BASF have prepared two patent applications (including EP 12183949.2) covering enzymatic processes for the synthesis of amines, and anticipate at least one more based on methodologies for functional oligosaccharide manufacture. GALAB has demonstrated the possibilities of glycosyltransferases for commercialized production of human milk oligosaccharides, with the potential to supply high-value, high quality products

for the improvement of infant formula milk. ENTR plans to offer halogenated compounds produced using the technology developed in BIONEXGEN via suppliers of fine chemicals.

The SME partners are uniquely placed to deliver the innovation required to implement academic knowledge, in response to the needs of industry. CLEA Technologies is a frontrunner in the development of green and cost-effective biocatalytic processes, delivering added value for customers that need to make the most of their raw materials and enabling them to meet market demands. The work of CLEA in BIONXGEN and beyond has demonstrated that biocatalysis affords substantial improvements in product quality, environmental impact and ultimately offers reductions in the cost of goods. Enzyme immobilization is a key enabling technology for the development of industrial biotransformations, facilitating the recycling and reuse of the biocatalyst, and CLEA is able to offer solutions to a wide variety of industries, ranging from fine chemicals and pharmaceuticals, cosmetics, flavours and fragrances to food and nutrition. Immobilized enzymes developed during BIONEXGEN will expand the product portfolio of CLEA, commercializing the *T. versicolor* Laccase CLEA as an initial offering resulting from this project.

The Joint Stock Company LentiKat´s a.s. (LK) is a small private Czech company (with 12 employees) manufacturing and supplying immobilized systems, “Lentikats Biocatalyst”, and related know-how, for industrial applications. As a result of the BIONEXGEN project, LK can offer two new products and new technologies to the market: firstly, immobilized laccase for the remediation of pollutants from wastewater; secondly, the application of α-L-Rhamnosidase (in the form of Lentikats Biocatalyst) for different applications, such as the enhancement of wine aromas.

Building on the Bidirectional Promoter technology developed in BONEXGEN and submitted as patent application in 2013, ACIB will form a new spin off company. This will create additional jobs in Austria and provide a reliable platform to access to this new technology by other European companies, in order to facilitate and enable their production processes. In addition the continued improvement of this new technology is expected to support further research and development work.

Wider Societal implications

New manufacturing routes to high-value and active compounds, such as chiral amines, lactones and lactams, halogenated small molecules, and flavonoids have the potential to improve quality of life for the whole of society. Production of high purity compounds, often possessing novel molecular architectures has the potential to lead to new advanced drug compounds with improved efficacy, also shortening development times, reducing pollution and hazardous by-product, and improving the efficiency of manufacturing. Access to cost-effective new therapies, and particularly ‘designer’ pharmaceuticals and ‘orphan’ drugs, will help to save and improve lives. These methodologies can be applied for the production of diverse classes of products, including flavours and fragrances, new monomers and polymers, and functional materials.

PUBLISHABLE SUMMARY Please provide a description of the potential impact (including the socio-economic impact and the wider societal implications of the project so far) and the main dissemination activities and the exploitation of results. The length of this part cannot exceed 10 pages.

Societal benefits from the shift to bio-based plastics could be enormous. Bio-based materials have the potential to produce fewer greenhouse gases, require less energy for production and recycling, and produce fewer toxic pollutants during their lifecycle than their fossil-based counterparts. Consumer demand is undoubtedly growing for functional and inexpensive green polymers, and the ongoing innovation supported by BIONEXGEN will further facilitate our steady conversion from the use of traditional polymers to sustainable, green materials.

New tools for manufacture of glyco-products will support research and development of glyco-based pharmaceuticals, improved infant formula nutrition, and functionalised materials for diverse applications. In particular, highly selective tools for manufacturing increasingly important therapeutic oligosaccharides and glycoproteins, and nutritional supplements will directly benefit human health during infant development and lead to improved treatments for disease. Functionalised materials derived from renewable plant-based resources have the potential to improve the environmental performance of consumer products by accessing sustainable feedstocks with reduced carbon emissions over the lifecycle of products, and to displace conventional hazardous and polluting chemistries.

Safer, greener manufacturing processes will improve working conditions for those employed in chemical manufacturing, and improve the lives of those living in proximity to production sites. The technologies developed in BIONEXGEN will help to ‘green’ the chemistry industry, not only by replacing polluting and energy-demanding technologies, but by improving the efficiency of existing processes by combining chemo-and biocatalysis, and applying biocatalytic technologies for the remediation of industrial waste streams.

Dissemination to the widest possible audience of scientific specialists, industrial end-users, policy makers, but importantly the European public, has been at the heart of BIONEXGEN. By participating in outreach activities, including open days for schools, public science fairs, and engaging with diverse media (including national newspapers, radio, and television), BIONEXGEN researchers have helped to increase awareness of EU research and industrial biotechnology. By contributing to the public understanding of industrial biotechnology, this project will help to drive consumer demand and acceptance of biocatalytic manufacturing for improved safety and environmental performance of consumer products. The enthusiastic interaction of BIONEXGEN scientists with young people and educators will help to increase the uptake of STEM subjects (Science, Technology, Engineering and Mathematics) by school children and students progressing to higher education, in order to nurture future generations of researchers, engineers, biocatalyst-end users, and chemical manufacturers.